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كلية الدراسات العليا

التاريـــخ: 8 / 5 / 2011

نموذج رقم (١٨)
اقرار والتزام بالمعايير الأخلاقية والأمانة العلمية
وقوانين الجامعة الأردنية وأنظمتها وتعليماتها
لطلبة الماجستير

أنا الطالبة: ايمان عمراني الرقم الجامعي: (8081453)
تخصص: صيدلة سريرية الكليـــــة: الصيدلة

عنوان الرسالة: Association between M2 polymorphism in CYP1A1 and Breast carcinoma in Jordanian Women: A Case-Control Study

اعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة باعداد رسائل الماجستير، عندما قمت شخصيا" باعداد رسالتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الرسائل العلمية. كما أنني أعلن بأن رسالتي هذه غير منقولة أو مستلة من رسائل أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة اعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بالغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

توقيع الطالب: Amrani التاريخ: 8 / 5 / 2011

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التوقيع ..... التاريخ ١١/٥/٨

ASSOCIATION BETWEEN *M2* POLYMORPHISM IN *CYP1A1* AND BREAST CARCINOMA IN JORDANIAN WOMEN: A CASE-CONTROL STUDY

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This Thesis was submitted in Partial Fulfillment of the Requirements for the Master's Degree of Clinical Pharmacy

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May, 2011

Committee Decision

This Thesis / Dissertation (ASSOCIATION BETWEEN *M2* POLYMORPHISM IN *CYP1A1* AND BREAST CARCINOMA IN JORDANIAN WOMEN: A CASE-CONTROL STUDY) was successfully defended and approved on 26th April, 2011.

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Dedication

My Very Special Family

You have been my number one believer and for you I am truly grateful. I deeply thank you for the unfailing love, support, humor and encouragement that made the completion of this degree possible.

❖

My great parents

I wish I could reward you half of what you offered to me during every step of my life, and what could really make me happy is to know that you are proud of me.

❖

My sister Lina, My brother Chems Eddine

You may be still teenagers, but you have always supported me in a way nobody else could, I wish I can offer you the love, the care and the support you need during the rest of your lives.

For you all I dedicate this humble work

Acknowledgement

First and foremost, I acknowledge that all of my opportunities and successes in life are gifts given to me in exceeding abundance from my God.

I would like to express my warm and sincere gratitude to my Supervisor Dr. Nailya Bulatova and my Co-supervisor Prof. Abdullah Abbadi for the continuous guidance, support, and motivation and insightful comments and corrections they gave me through the whole road of this research. Their kindness, patience and sincere feelings made me feel at home.

I am grateful to the committee members for devoting their time to read and discuss this thesis and for providing valuable comments for its improvement.

I owe my deep thanks to Dr. Al- Motassem Yousef for his valuable help through the final steps of my work.

During my work I have collaborated with many health care professionals (especially Dr. Mahmoud Al Masri, Dr. Jamal Masaad, and Dr. Laila Abu Tahoun) for whom I have great regard. I have been blessed to be surrounded by such kind and generous people while collecting samples from JUH, KHCC, and Jordan hospital.

I heartily offer my regards and blessings to the head (Dr. Said Ismail) and team of Molecular Biology Research Laboratory at JU for introducing me to this field, without your valuable technical help, it wouldn't be possible to perform such work and without your sweet friendship it wouldn't be possible to handle the difficulties I encountered during that period.

The professors in the faculty of Pharmacy at Jordan University have provided an exceptional education, and I like to thank them for that and for the permanent encouragement and confidence in me as a graduate student. I really appreciate it.

Finally, I would like to express my deep gratitude to Jordan for its hospitality and to my country Algeria, for this opportunity.

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List of Abbreviations

Abbreviation	Definition
3'-UTR	Three Prime Untranslated Region
A	Adenine Nucleotide
AA	Aromatic Amine
AHH	Aryl Hydrocarbon Hydroxylase
AhR	Aryl Hydrocarbon Receptor
ARNT	Aryl hydrocarbon Receptor Nuclear Translocator
Asp	Asparagine
ATM	Ataxia Telangiectasia Mutated
B[*a*]P	Benzo alpha pyrene
BI-RADS	The Breast Imaging Reporting and Data System
BMI	Body Mass Index
bp	Base Pair
BRCA1	Breast Cancer 1, early onset gene (or protein)
BRCA2	Breast Cancer 2, early onset gene (or protein)
BSA	Bovine Serum Albumin
C	Cytosine Nucleotide
CE	Catechol estrogen
CI	Confidence Interval
CMF	Cyclophosphamide, Methotrexate, Fluorouracil
COMT	Catechol-O-Methyltransferase
CTP	Deoxycytidine Triphosphate
CYP	Cytochrome P450
CYP1A1	Cytochrome P450 1A1

dATP	Deoxyadenosine Triphosphate
DCIS	Ductal Carcinoma In Situ
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
dNTP	Desoxyribonucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen Receptor
FFTP	first full time pregnancy
G	Guanine Nucleotide
GSH	Glutathione
GST	Glutathione S-Transferase
GTP	Deoxyguanosine Triphosphate
HAA	Heterocyclic Aromatic Amine
HER-2	Human Epidermal growth factor Receptor 2
HR	Hazard Ratio
HRT	Hormone Replacement Therapy
H-W	Hadry-Weinberg
Ile	Isoleucine
JUH	Jordan University Hospital
KHCC	King Hussein Cancer Center
LCIS	Lobular Carcinoma In Situ
M	Metastasis
M	Mutation
MeOE	Methoxyestrogens

$MgCl_2$	Magnesium chloride
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
N	Lymph Node involvement
NAT	*N-acetyltransferases*
NFW	Nuclease free water
NOS	No Specific Type
OHE	Catechol estrogen
OR	Odds Ratio
P	Probability Value
p53	Tumor Protein 53
PAH	polycyclic aromatic hydrocarbon
PCB	Polychlorinated Biphenyl
PCR	Polymerase Chain Reaction
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin Homolog
Q	Long Arm of the Chromosome
RFLP	Restriction Fragment Length Polymorphism
RR	Relative Risk
rs	Reference SNP ID
SIR	Standardized Incidence Ratio
SNP	Single Nucleotide Polymorphism
SQ	Semiquinone
T	Tumour size

T	Thymine Nucleotide
TBE	Tris-Borate-EDTA
Thr	Threonine
TM	Melting Temperature
TTP	Deoxythymidine Triphosphate
UK	United Kingdom
US	United States
Val	Valine
WBC	White Blood Cell
WHR	Waist to Hip Circumferences Ratio
χ^2	Chi-square

ASSOCIATION BETWEEN *M2* POLYMORPHISM IN *CYP1A1* AND BREAST CARCINOMA IN JORDANIAN WOMEN: A CASE-CONTROL STUDY

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ABSTRACT

CYP1A1 is a candidate gene for low-penetrance breast cancer susceptibility, as it plays an important role in phase I metabolism of carcinogens such as polycyclic aromatic hydrocarbons (PAHs), as well as in the oxidative metabolism of estrogens, the hormones that play a critical role in the etiology of breast carcinoma. Many studies have shown that *CYP1A1* polymorphisms were significantly associated with breast carcinoma risk, whereas others found no association. The objective of this study was to assess the association between *M2 (A4889G)* polymorphism in *CYP1A1* and breast cancer risk among Jordanian women and in subgroups stratified by histopathological type, hormone receptor status, smoking history and menopausal status. Blood samples were collected from 2 groups of Jordanian women: 112 breast cancer female patients and 115 age-matched controls who underwent mammography with or without breast ultrasound and have shown negative results (BI-RADS I or BI-RADS II). Participants were also interviewed to obtain data on demographic, reproductive, hormonal, lifestyle, and environmental risk factors of breast cancer. Patients and controls were genotyped for *M2 (A4889G)* polymorphism in *CYP1A1*using Polymerase Chain Reaction- Restriction Fragment Length Polymerase (PCR-RFLP) analysis. Among 112 breast cancer patients, 102 (91.07%) were homozygote wild type (*AA*), 10 (8.93%) were heterozygote variant type (*AG*) and none (0%) was homozygote variant type (*GG*). Among the 115 controls, 102 (88.70%) were homozygote wild type (*AA*), 12 (10.43%) were heterozygote variant type (*AG*) and only 1 (0.87%) was homozygote variant type (*GG).* No statistically significant overall association was found between *M2 CYP1A1* genotypes and breast cancer risk (*p*- value = 0.55; OR = 0.77; 95% CI= 0.32 – 1.83). Analysis of subgroups defined by menopausal status, smoking history, and oral contraceptive use has also shown no association with *M2 CYP1A1* genotype. Noteworthy, physical activity was associated with 60 % (OR= 0.4; 95% CI= 0.21-0.77; *p* = 0.006) reduced breast cancer risk. This effect was more pronounced among premenopausal women. In addition, postmenopausal obesity and the physical trauma to the breast were associated with statistically significant increased breast cancer risk (*p*=0.001 and 0.04, respectively). **Conclusion**: based on the study results, *CYP1A1 M2* polymorphism does not

play a major role in breast cancer risk among Jordanian females, either alone or in combination with environmental or hormonal factors. However, because of the rarity of this polymorphism among Jordanian women, larger studies are needed to confirm or refute these findings.

I. INTRODUCTION

Breast cancer is the most common female cancer worldwide, the second most common cause of cancer death in women in the US, and the main cause of death in women aged 40 to 59 (Hayes, 2010) . The etiology of breast carcinoma is still poorly understood in spite of known breast carcinoma risk factors such as age, reproductive events (menarche, menopause, pregnancy, breastfeeding), exogenous hormones (hormone replacement therapy and oral contraceptives), lifestyle and environmental risk factors (diet, obesity pollution, alcohol), ionizing radiation, as well as genetic factors (high and low-penetrance breast cancer susceptibility genes) (Khedhaier A, *et al.*, 2008). In hereditary breast carcinoma, mutations in high-penetrance genes such as *BRCA1* or *BRCA2* confer a relatively high risk for developing breast cancer, though this risk accounts only for about 5 to 10% of all breast carcinoma cases (Bennett, *et al*., 1999). Therefore, it is suggested that the effect of low-penetrance cancer susceptibility genes modulated by environmental exposure and lifestyle factors are likely to account for most of sporadic breast carcinoma cases. The proportion of breast carcinoma cases attributable to such genetic traits, in combination with environmental exposure, is likely to be much higher than the hereditary proportion and accounts for 90 to 95% of all breast carcinoma cases (Khedhaier A, *et al.*, 2008). *CYP1A1* is a candidate gene for low-penetrance breast cancer susceptibility because it plays an important role in phase I metabolism of carcinogens such as polycyclic aromatic hydrocarbons (PAHs) as well as in the oxidative metabolism of estrogen, the hormone that plays a critical role in the etiology of breast carcinoma (Basham, *et al.*, 2001). PAHs are known human carcinogens, are present in tobacco smoke and ubiquitous in urban environments, and they have been known to cause mammary tumors in rodents (Cavalieri

N, *et al.*, 1988). *CYP1A1* gene is highly polymorphic, and the polymorphisms in this gene have been suggested as risk factors for breast cancer in some populations (Zhang, *et al.*, 2004; Surekha, *et al.*, 2009). Understanding one of the risk factors for breast cancer permits us to identify women at increased risk and intervene to modify this risk, both individually and collectively (Costanza and Chen, 2010).

1. Prevalence of breast cancer:

Globally, breast cancer incidence rates are highest in North America and northern Europe and lowest in Asia and Africa. These international differences are thought to be related to societal changes occurring during industrialization (e.g., changes in fat intake, body weight, age at menarche, and/or lactation, and reproductive patterns such as fewer pregnancies and later age at first birth) (Costanza and Chen, 2010).

In Jordan, breast cancer ranked first among cancers in females and it accounted for 36.7% of all female cancers (Figure 1) In 2008, there were 864 breast cancer cases among Jordanians, accounting for 18.8 % of all newly diagnosed cancer cases. There were 855 female breast cancer cases. Only 9 cases of breast cancer were diagnosed in males resulting in a female to male ratio of 95:1. The crude incidence rate for female breast cancer was (30.2) per 100,000 female population, compared to (29.5) per 100,000 in 2007. (Jordan's Ministry of Health, 2008).

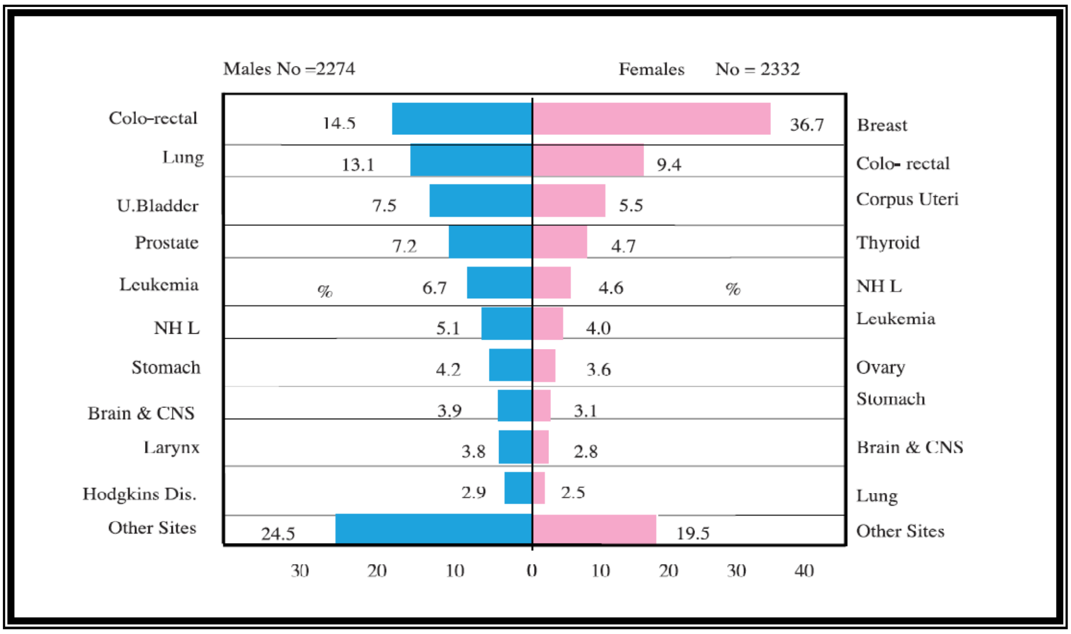


Figure 1: The percentage distribution of the most frequent types of cancer by gender (all ages) in 2008 (Jordan's Ministry of Health, 2008).

The median age at diagnosis of breast cancer in Jordanian females was 51 years, ranging from 22 years to 91 years. About 30.2% of cases were in the age group 40-49 years, while 24% of the cases were in the age group 50-59 years. Only 2.2% were under 30 years and 4.7 % were above 75 years. Jordanian women are afflicted with breast cancer at a much younger age (median age is 51) than women in Western countries (median age is 65), when they are still raising children, caring for their families, and contributing to the growth and development of society (Jordan's Ministry of Health, 2008).

Till 2007, the mortality rate has decreased from 24.3% (in 2006 according to Jordan's Ministry of Health, 2007) to 22.3% in breast cancer female patients (Jordan's Ministry of Health, 2008) (Table 1). Survival rates and the early detection of breast cancer are directly

connected; yet fortunately, public awareness in Jordan regarding this fact is increasing (Jordan's Ministry of Health, 2008).

Table 1: Distribution of female cancer deaths in Jordan, 2007 (Jordan Ministry of health, 2008).

Cancer	Number	%
Breast	197	**22.3**
Leukemia	94	10.6
Colorectal	83	9.4
Uterus and cervix	63	7.1
Lung	53	6
NHL	51	5.8
Liver	38	4.3
Pancreas	36	4.1
Ovary	32	3.6
Stomach	30	3.4
Others	69	7.8
Unknown	137	15.6
Total	883	100

2. Pathogenesis:

The breast undergoes dramatic changes in size, shape, and function in association with puberty, pregnancy, and lactation. The risk of developing breast cancer has been linked to both endogenous and exogenous hormonal influences, as well as environmental and genetic factors. The molecular mechanisms underlying the development of breast cancer, particularly estrogen-associated breast carcinogenesis, are incompletely understood (Russo, 2010).

3. Breast Disorders:

Intrinsic disorders of the breast are either malignant (breast cancer) or benign (e.g., fibrocystic disease). Breast disease also can occur as a result of the effects of other disorders or drug therapy, as in galactorrhea.

3.1. Benign Breast disorders:

In women, the breast, like other estrogen- and progesterone-responsive tissues, displays cyclic changes in concert with alterations in the level of ovarian steroids through the menstrual cycle. Subtle imbalances in the relative levels of estrogen and progesterone may be the cause of so-called benign breast disease. This term refers to abnormalities ranging from normal premenstrual breast tenderness relieved with menstruation at one extreme to so-called fibrocystic disease at the other. In fibrocystic disease, breast fibrosis and cysts are associated with mammary epithelial hyperplasia. Normal breast tissue may have either fibrosis or cysts but not epithelial cell hyperplasia. True fibrocystic disease with epithelial cell hyperplasia is a risk factor for breast cancer in much the same way that endometrial hyperplasia resulting from unopposed estrogen action is a risk factor for endometrial cancer. Benign epithelial breast lesions can be classified histologically into three categories: nonproliferative, proliferative without atypia, and atypical hyperplasia. The categorization is based upon the degree of cellular proliferation and atypia (Purcell and Taylor, 2009).

3.2. Breast Carcinoma:

3.2.1. Diagnosis:

Initial workup for a woman presenting with a lesion or symptoms suggestive of breast cancer should include a careful history, physical examination of the breast, three-dimensional mammography, and possibly other breast imaging techniques such as ultrasonography or MRI. Most breast cancers can be visualized on a mammogram as a mass, a cluster of calcifications, or a combination. The Breast Imaging Reporting and Data System (BI-RADS) was developed by the American College of Radiology to standardize mammographic reporting (Michaud, *et al.*, 2008). Table 2 presents BI-RADS classifications and management recommendations. Early detection and treatment improve prognosis and survival rate, motivating the implementation of screening tests and improved methods of staging therapy (Michaud, *et al.*, 2008).

Table 2: BI-RADS Categories and Management Recommendations for Mammograms (Costanza and Chen, 2010).

BI-RADS category	Assessment	Recommendations
0	Assessment is incomplete	Need additional imaging evaluation and / or mammograms for comparison
1	Negative	Continue routine screening
2	Benign findings (cysts, benign calcifications, ...)	Continue routine screening
3	Probably benign findings (likelihood of malignancy is less than 2 %)	Initial short interval follow-up suggested (6 months)
4	Suspicious abnormality	Biopsy should be considered
5	Highly suggestive of malignancy	Appropriate action should be taken; biopsy or treatment as indicated
6	Biopsy-proven malignancy	Appropriate action should be taken; evaluate the contralateral breast, or assess response to neoadjuvant chemotherapy

3.2.2. Histopathology :

More than 95 percent of breast malignancies arise from the breast epithelial elements and are therefore carcinomas (Bleiweiss, 2010). The breast carcinomas consist of several histologic subtypes:

3.2.2.1. Invasive breast carcinomas (Bleiweiss, 2010):

- Infiltrating ductal carcinoma is the most common type of invasive breast cancer, accounting for 70 to 80 percent of invasive cancers.
- Infiltrating lobular carcinoma is the second most common invasive breast cancer, accounting for 5 to 10 percent of invasive cancers.

Other less common invasive breast carcinoma histologies include tubular, mucinous, and medullary carcinomas.

3.2.2.2. In situ Carcinomas (Bleiweiss, 2005):

These lesions confine themselves to the ducts or lobules and do not spread to the surrounding tissues in the breast or other parts of the body.

- Ductal Carcinoma in Situ (DCIS) is characterized by a proliferation of abnormal cells confined within the mammary ductal system.
- Lobular Carcinoma in Situ (LCIS) represents abnormal tissue growth that occurs predominantly within the terminal duct-lobular units.

In Jordan, the histopathological distribution of female breast cancers showed that 84.7 % were infiltrating ductal carcinoma , 4.8 % Lobular carcinoma, 1.3% were mixed ductal and lobular carcinoma , carcinoma of No Specific Type (NOS) 3.5 %, the remaining (5.7%) were other types of morphology (Jordan's Ministry of Health, 2008).

3.2.3. Staging (Michaud, *et al.*, 2008):

Stage is defined on the basis of the primary tumor extent and size (T1–4), presence and extent of lymph node involvement (N1–3), and presence or absence of distant metastases (M0–1).

1. The stage 0 represents carcinoma in situ (Tis) or disease that has not invaded the basement membrane of the breast tissue (Tis N0 M0).

2. Stage I represents a small primary invasive tumour without lymph node involvement (T1 N0 M0, T0 N1mi M0 and T1 N1mi M0).

3. Stage II disease usually involves regional lymph nodes. Stages I and II are often referred to as early breast cancer. It is in these early stages that the disease is highly curable (Stage IIA: T0 N1 M0, T1 N1 M0, T2 N0 M0; Stage IIB: T2 N1 M0 and T3 N0 M0).

4. Stage III, also referred to as locally advanced disease, usually represents a large tumour with extensive nodal involvement in which either node or tumour is fixed to the chest wall (Stage IIIA: T0 N2 M0, T1 N2 M0, T2 N2 M0, T3 N1 M0, T3 N2 M0; Stage IIIB: T4 N0 M0, T4 N1 M0, T4 N2 M0; Stage IIIC: Any T N3 M0).

5. Stage IV disease is characterized by the presence of metastases to organs distant from the primary tumour and is often referred to as advanced or metastatic disease as described earlier (Any T Any N M1).

In Jordan, 55% percent of breast cancer cases in the country are presented at early stages (0-II) during which survival rates are high and the disease is more curable and only 37% of cases are diagnosed in advanced stages (III-IV) and the rest 8% were of undefined stage at diagnosis. This is a reverse statistic in 2005 and the positive data are due to screening programs that helped in the diagnosis of the disease in its early stages (Jordan's Ministry of Health, 2008).

3.2.4. Other tumour characteristics:

A variety of factors influence the treatment of localized breast cancer because they have a bearing on local treatment, prognosis, and selection of adjuvant treatment. The most important are the stage (lymph node involvement, tumour size), and hormone receptors and/or the protein HER-2 expressed by the tumour. (Hayes, 2010).

3.2.4.1. Hormone receptors:

Assay of hormone receptors (estrogen [ER] and progesterone [PR] receptors) is an important component of the pathologic evaluation of a resected breast cancer. Women with hormone receptor-positive tumors benefit from the addition of postoperative endocrine treatments such as tamoxifen, and in postmenopausal women, the aromatase inhibitors (anastrozole [Arimidex®], letrozole [Femara®], exemestane [Aromasin®]), or for premenopausal women, ovarian ablation or suppression.(Hayes, 2010).

3.2.4.2. HER-2 overexpression:

All primary tumors should be assayed for HER2 expression and approximately 20% have high levels of overexpression. High levels of HER2 expression represent an important predictive factor, identifying those patients who might benefit from treatments that target HER2, such as Trastuzumab, both in the adjuvant and metastatic disease settings. In addition, HER2 overexpression is also a predictive factor for response to anthracycline-based as compared to CMF (cyclophosphamide, methotrexate and fluorouracil)-type adjuvant chemotherapy (Hayes, 2010).

4. Breast cancer Risk Factors:

4.1. Age/Gender :

Age and gender are among the strongest risk factors for breast cancer. Breast cancer occurs 100 times more frequently in women than in men. The incidence rises throughout a woman's lifetime, until about the age of 45 to 50 when the rise is less steep (The change in slope probably reflects the impact of hormonal change (menopause) that occurs about this time), and peaking at the age of 75–80 years and then declining slightly thereafter. Breast cancer is very rare in all ethnic groups before the age of 25 (Costanza and Chen, 2010).

4.2. Race/Ethnicity :

According to the American Cancer Society, the highest rates occur in whites (133 cases per 100,000 women) and the lowest in American Indians/Alaska natives (70 per 100,000). Much of these ethnic differences are attributable to factors associated with lifestyle and socioeconomic status (e.g., access to diagnosis and treatment). Genetic and/or biologic factors also may contribute (Costanza and Chen, 2010).

4.3. Benign Breast Disease:

Single nonproliferative lesions (fibrocystic change, solitary papilloma, simple fibroadenoma) are not associated with an increased risk for breast cancer. The presence of multiple nonproliferative lesions increased the risk for breast cancer modestly (RR= 1.8; 95% CI=1.20-2.70) at 10 years in one cohort study (Worsham, *et al.*, 2007). The more

important precursors of noninvasive or invasive breast cancer are proliferative lesions, particularly those with cytologic atypia (complex fibroadenoma, atypical hyperplasia) (Degnim, *et al.*, 2007).

4.4. Personal history of breast cancer:

A personal history of invasive or in situ breast cancer increases the risk of developing an invasive breast cancer in the contralateral breast (Costanza and Chen, 2010).

4.5. Lifestyle and dietary factors

4.5.1. Socioeconomic level:

Women of higher socioeconomic status are at greater risk for breast cancer (SIR=1.40, 95% CI= 1.35–1.46 for the highest strata). However, it does not appear that socioeconomic status is an independent risk factor. Instead, the influence of socioeconomic status (educational, occupational, and economic level) is thought to reflect differing reproductive patterns with respect to parity, age at first birth, age at menarche, and utilization of screening mammography (Pukkala and Weiderpass, 1999).

4.5.2. Geographic residence:

There are marked variations in breast cancer incidence and mortality among countries. Globally, breast cancer incidence rates are highest in North America and northern Europe

and lowest in Asia and Africa. These international differences are thought to be related to societal changes occurring during industrialization (e.g., changes in fat intake, body weight, age at menarche, and/or lactation, and reproductive patterns such as fewer pregnancies and later age at first birth) (Costanza and Chen, 2010).

4.5.3. Body size:

Weight and body mass index (BMI) have opposite influences on postmenopausal as compared to premenopausal breast cancer. There is decreased risk in obese women younger than 40 years as a result of the association with anovulatory cycles and lower progesterone levels late in the cycle. In a previously described pooled analysis of seven prospective cohort studies, premenopausal women with a BMI ≥ 31 kg/m^2 were 46 percent less likely to develop breast cancer than those with a BMI <21 kg/m^2 (Brandt, *et al.*, 2000). In contrast, the risk is increased for postmenopausal obese women, which is attributed to the synthesis of estrogens in fat depots (Feigelson, *et al.*, 2004). However, the prevailing theory suggests a more important role for the localization of body fat measured as waist-to-hip circumferences ratio (WHR) (Mannisto, *et al.*, 1996).

4.5.4. Physical activity:

Regular physical exercise appears to provide modest protection against breast cancer, but the relationship is complex, particularly in premenopausal women. Some studies have shown a decreased risk of premenopausal breast cancer in women who exercise more, particularly during adolescence (Maruti, *et al.*, 2008), but others have shown no difference (Mertens, *et al.*, 2006**)**. In premenopausal women, even moderate physical activity can be

associated with anovulatory cycles, which are associated with decreased risk. On the other hand, thinner premenopausal women have a higher risk of breast cancer than do heavier women (Brandt, *et al.*, 2000). Among postmenopausal women, the literature more consistently shows a protective effect of regular strenuous activity on breast cancer incidence (McTiernan, *et al.*, 2003).

4.5.5. Dietary Factors:

4.5.5.1. Alcohol:

More than 40 epidemiologic studies have evaluated a possible association between alcohol intake and breast cancer. Taken together, there is consistent evidence that breast cancer risk is higher for women consuming moderate to high levels of alcohol (≥three drinks/day) compared with abstainers. A number of biological mechanisms may explain the association between breast cancer and alcohol intake, including increased circulating estrogens and androgens, enhancement of mammary gland susceptibility to carcinogenesis, increased mammary carcinogen DNA damage, and greater potential for invasiveness of breast cancer cells (Singletary and Gapstur, 2001; Allen, *et al.*, 2009).

4.5.5.2. Fat intake:

Despite intense interest in dietary fat as a risk factor for breast cancer, the studies have been inconsistent, and for those that show a positive association, the effect has been modest. In contrast,

the data linking postmenopausal BMI and weight gain with a higher risk of breast cancer have been consistent and demonstrate a stronger association (Costanza and Chen 2010).

4.5.6. Smoking:

Studies have shown widely varying results, with many showing modestly increased risk. One explanation for the inconsistencies in the results may be that the first generation studies evaluated associations of smoking with breast cancer by comparing active smokers to non-active smokers (Morabia, 2002), while the second generation studies separated passive smokers from the reference groups (Khuder and Simon, 2000). The composition of sidestream smoke is different from mainstream smoke, e.g., containing higher levels of nitrosamines and PAHs, thus further emphasizing the importance of separating passive smokers from the reference group. Cigarette smoke could be expected to increase the risk of breast cancer as it is known to contain about 70 carcinogens (e.g., polycyclic aromatic hydrocarbons (PAHs), aromatic amines (AAs), heterocyclic aromatic amines (HAAs) and N-nitrosamines) out of about 4800 identified compounds (Hoffman, *et al.*, 2001). Many of those carcinogens have been shown to induce mammary tumours in rodents (Cavalieri N, *et al.*, 1988). Furthermore, smoking related DNA adducts have been found in the breast tissue of smokers (Li, *et al.*, 1996). Recently, a prospective cohort study of 111,140 participants by Xue, *et al.* (2011) has shown that breast cancer incidence was associated with a higher quantity of current and past smoking, younger age at smoking initiation, longer duration of smoking, and more pack-years of smoking. Premenopausal smoking was associated with a slightly higher incidence of breast cancer (HR, 1.11; 95% CI, 1.07-1.15 for every increase of 20 pack-years), especially smoking before first delivery (HR =1.18; 95%CI= 1.10-1.27

for every increase of 20 pack-years). Conversely, the direction of the association between postmenopausal smoking and breast cancer was inverse (HR =0.93; 95%CI =0.85-1.02 for every increase of 20 pack-years).

4.6. Reproductive / hormonal risk factors

Prolonged exposure to and high concentrations of endogenous and exogenous estrogen increases the risk of breast cancer (Costanza and Chen, 2010).

4.6.1. Endogenous estrogens:

Lifetime exposure to endogenous sex hormones is determined by several variables including: age at menarche, age at first full time pregnancy (FFTP), number of pregnancies, and age at menopause.

4.6.1.1. Age at menarche:

Younger age at menarche is associated with a higher risk of breast cancer (Hsieh, *et al.*, 1990; Berkey, *et al.*, 1999). In one study, for every two-year delay in the onset of menarche, there was a 10 percent reduction in cancer risk (Hsieh, *et al.*, 1990). Cumulative lifetime estrogen exposure may explain the association between the age of menarche and breast cancer.

4.6.1.2. Age at menopause:

Later menopause increases breast cancer risk. The relative risk increases by 1.03% for each year older at menopause, which is comparable to the increase with hormone replacement therapy (HRT) use (Beral, *et al.*, 1997).

4.6.1.3. Parity:

A metaanalysis of eight Nordic studies showed that nulliparous women were at 30% increased risk of breast cancer compared to parous women (Ewertz, *et al.*, 1990). The protective effect of pregnancy is not seen until after 10 years following delivery (Bruzzi, *et al.*, 1988). Whether multiparity confers protection against breast cancer has been a matter of controversy, the majority of studies suggest a decreased risk with increasing number of pregnancies (Kelsey and Gammon, 1993; Pathak, *et al.*, 2000).

4.6.1.4. Age at first full term pregnancy:

The younger a woman is at her first full-term pregnancy, the lower her breast cancer risk, In the Nurses' Health Study, the cumulative incidence of breast cancer up to age 70 for parous versus nulliparous women was 20 percent lower if the first birth was at age 20, 10 percent lower for first birth at age 25, and 5 percent higher if the first birth was at age 35 (Rosner, *et al.*, 1994). The mechanism of the protective effect of parity is not completely understood, but probably reflects early full differentiation of mammary gland cells, rendering them less susceptible to carcinogenic transformations (Russo, *et al.*, 2000).

4.6.1.5. Abortion:

Both a large pooled analysis (Beral, *et al.*, 2004) and population-based cohort studies (Paoletti and Clavel-Chapelon, 2003; Michels, *et al.*, 2007) do not support an association between abortion (induced or spontaneous) and breast cancer risk.

4.6.1.6. Breastfeeding:

A protective effect of breastfeeding has been shown in multiple case-control and cohort studies. A large pooled analysis that included individual data from 47 epidemiologic studies including 50,302 women with invasive breast cancer and 96,973 controls estimated that the relative risk of breast cancer was reduced by 4.3% for every 12 months of breastfeeding, in addition to a decrease of 7 percent for each birth (Beral, *et al.*, 2002). A postulated mechanism for the protective effect of breastfeeding is that it may delay the reestablishment of ovulatory cycles. Other mechanisms may be the increase in prolactin secretion and the concomitant decrease in estrogen production (Costanza and Chen, 2010).

4.6.2. Exogenous hormone factors:

4.6.2.1. Oral contraceptives:

Many epidemiologic studies have failed to demonstrate an association between oral contraceptive use and the risk of breast cancer. However, a large pooled analysis calculated a small but significant increase in relative risk of breast cancer (RR =1.24, 95% = 1.15-1.33) in current oral contraceptive users (Beral, *et al.*, 1996). Concerns have been raised

about this analysis because a low percentage of women had ever used oral contraceptives (40 percent), and it lacked the follow-up necessary to determine whether there were long-term effects of oral contraceptive use. There are now reassuring data from two studies (Hankinson, *et al.*, 1997; Marchbanks, *et al.*, 2002) that oral contraceptives do not increase breast cancer risk later in life (Costanza and Chen, 2010).

4.6.2.2. HRT:

The bulk of the currently available evidence supports a causal relationship between the use of postmenopausal HRT and breast cancer, predominantly hormone receptor-positive breast cancer. A reanalysis of original data from 51 epidemiologic studies comprising 52,705 women with and 108,411 women without breast cancer found that for each year a woman uses postmenopausal hormones, her risk of breast cancer increases by 2.3 % and a 35% increase in the risk was seen for women who had used HRT for 5 years or longer (Beral, *et al.*, 1997). The combined estrogen–progestin regimen is associated with greater increases in the risk than estrogen alone (Schairer, *et al.*, 2000).

4.6.2.3. Xeno-estrogens:

Xeno-oestrogens, which include pesticides, dyes, pollutants, plasticizers and food preservatives that have oestrogen-like effects; have been suggested to have a role in the etiology of breast cancer (Davis, *et al.*, 1993; Dees, *et al.*, 1997; Safe, 1997). Xeno-oestrogens have also been called endocrine disrupters because they interfere with the actions of endogenous estrogens. For instance, catechol metabolites of the organochlorins

such as polychlorinated biphenyls (PCBs) have been suggested to alter oestrogen metabolism by inhibiting the inactivation of carcinogenic estrogen metabolites. A comprehensive review of dichlorodiphenyltrichloroethane (DDT) did not, however, support the association between this compound and breast cancer risk (Snedeker, 2001) and most large studies have failed to find an association (Calle, *et al.*, 2002). A breast cancer case–control study provided the first demonstration of a significant relationship between breast cancer risk and estrogenicity due to xenoestrogens taking into account the environmental, dietary, lifestyle, genetic and reproductive factors, which are not usually systematically measured across studies. (Ibarluzea, *et al.*, 2004).

4.7. Family history:

Family history is an important risk factor for breast cancer. However, a positive family history is only reported by 15 to 20 percent of women with breast cancer (Costanza and Chen, 2010). The risk associated with having an affected first or second degree maternal or paternal relative is modulated by the age of both the case patient and the family member at diagnosis, and the number of female first-degree relatives with and without cancer. As an example, in a pooled analysis using data from over 50,000 women with breast cancer and 100,000 controls, the risk of breast cancer for a woman with one affected first-degree relative was increased 1.80 fold (95% CI= 1.69-1.91). With two affected first-degree relatives, the risk was increased 2.93 fold (95% CI= 2.36-3.64). The risk ratios were highest for women with young affected relatives. Thus, the risk in patients younger than 50 was increased 2.9- fold (95% CI =1.9–4.4) for a woman whose relative was diagnosed before age 40, but only 2.0-fold (95% =1.2–3.2) if the affected relative was diagnosed after age 60. Similarly,

the risk of breast cancer before age 40 was increased 5.7-fold if one relative had breast cancer before age 40 (Beral, *et al.*, 2001).

4.8. Genetic mutations and polymorphisms:

The molecular mechanisms underlying the development of breast cancer are not completely understood. However, it is generally believed that the initiation of breast cancer is a consequence of cumulative genetic damages leading to genetic alterations that result in activation of proto-oncogenes and inactivation of tumour suppressor genes. These in turn are followed by uncontrolled cellular proliferation and/or aberrant programmed cell death, or apoptosis (Mitrunen and Hirvonen, 2003). Epidemiological and human genetic studies have identified two classes of susceptibility genes:

4.8.1. High penetrance genes:

It has been estimated, that around 5–10% of breast cancer cases are caused by germline mutations in breast cancer susceptibility genes (Claus, *et al.*, 1996). Two major breast cancer susceptibility genes, *BRCA1* and *BRCA2* have been identified on chromosomes 17q and 13q, respectively (Miki, *et al.*, 1994, Wooster, *et al.*, 1995). *BRCA1* and *BRCA2* are so called high penetrance genes, leading to a high probability of developing breast cancer among carriers of the variant alleles of these genes. The proteins encoded by these genes, which are suggested to act as tumour suppressors, are involved in the maintenance of genomic integrity. They participate in repairing DNA damage by homologous recombination and are also proposed to regulate transcription (Scully, 2000; Tutt and Ashworth, 2002). Other well known high-penetrant genes include: tumor protein 53 gene

(*p53)*, Ataxia Telangiectasia Mutated gene *(ATM)*, and Phosphatase and Tensin Homolog gene *(PTEN)*. The high penetrance of these autosomal dominant inherited conditions results in multiple cases of cancers among first and second degree relatives, generally at a younger age (Kotnis, *et al.*, 2005).

4.8.2. Low penetrance genes:

As disease-causing allelic variants of the high-penetrance genes are expected to be relatively rare in the general population, the proportion of breast cancer in the population that may be explained by these genotypes is likely to be much lower than that of the low-penetrance genes. In contrast to familial cases, low penetrance genes contribute to sporadic cases of breast cancer that usually appear unilaterally, and have a relatively late age at diagnosis (Rebbeck, 1999). These tumors are likely to be caused by the interaction between many genetic and environmental factors (Mitrunen and Hirvonen, 2003).

In general, the search for low-penetrance susceptibility genes relies on knowledge about biochemical or physiological pathways that are thought to affect breast carcinogenesis. Candidate polymorphic genes include those encoding for enzymes involved in oestrogen and carcinogen metabolism, and in the detoxification of reactive oxygen species emerging in these reactions (Figure 2). Among the most widely studied low-penetrance genes in relation to breast cancer are those encoding for cytochromes P450 (CYPs), glutathione S-transferases (GSTs), N-acetyltransferases (NATs) and catechol-O-methyltransferase (COMT). (Dunning, *et al.*, 1999; Kristensen and Borresen-Dale, 2000).

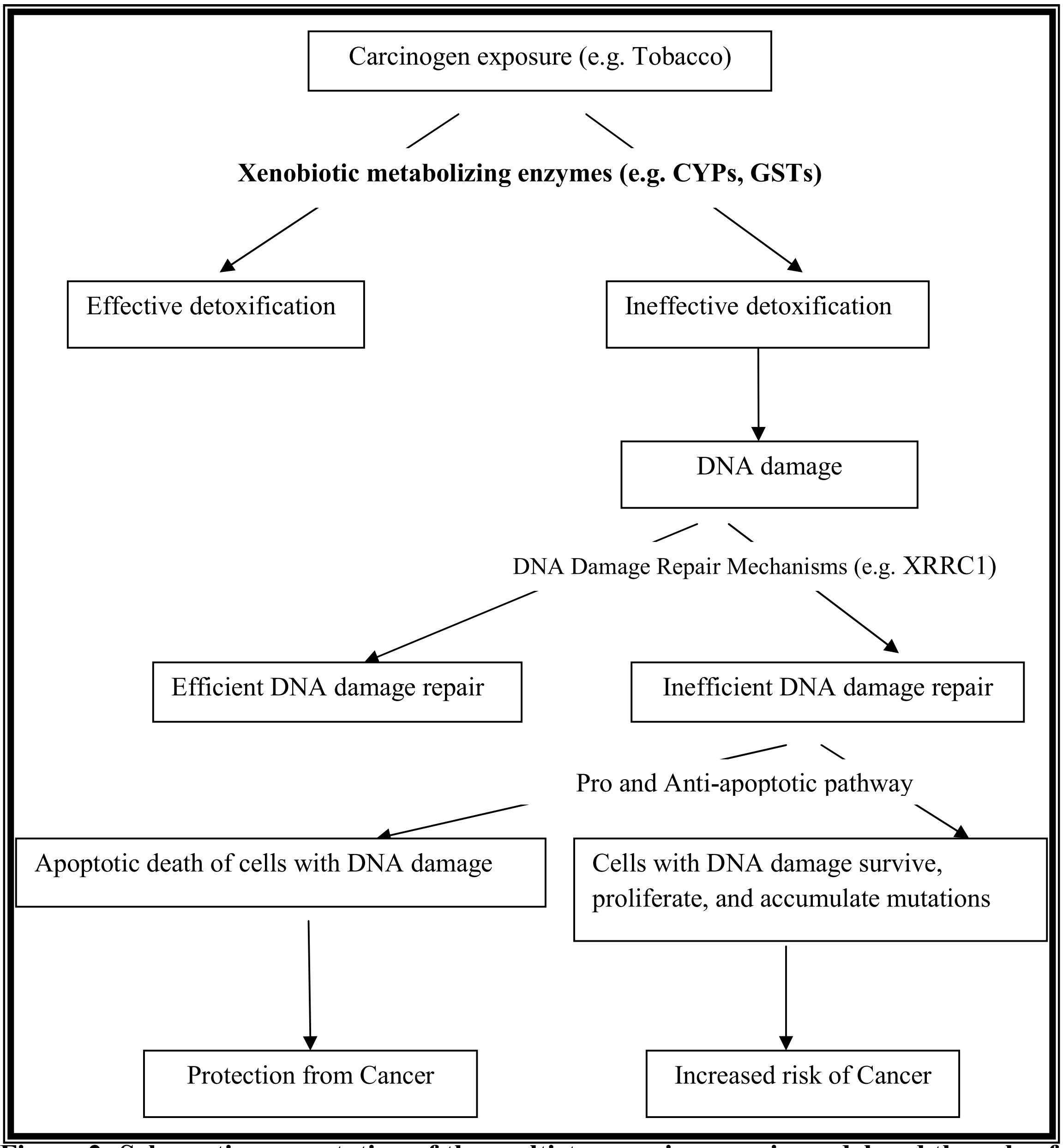


Figure 2: Schematic presentation of the multistep carcinogenesis model and the role of low penetrance genes (Kotnis, *et al.*, 2005).

5. CYPs:

The phase-I activating enzymes called Cytochrome P450 isoenzymes (CYPs) are a superfamily of haemoprotein enzymes found on the membrane of endoplasmic reticulum. They are responsible for catalyzing the metabolism of large number of endogenous and

exogenous compounds. CYPs are also known as mixed function oxidases or mono-oxygenases as metabolism of a substrate by a CYP consumes one molecule of molecular oxygen and produces an oxidized substrate and another molecule of oxygen appears in water as byproduct. Cytochrome P450 isoenzymes are predominantly present in liver but are also found in intestine, lungs, kidneys, brain, breast, etc. Different CYPs are important in human metabolism of xenobiotics. Cytochrome P450 isoenzymes are designated by the letters e.g., CYP2D6 (CYP followed by an Arabic numeral, a letter and another Arabic numeral). The "P" in cytochrome P450 stands for 'pigment'. These enzymes have a spectrophotometric absorption peak at or near 450 nm when bound and reduced by carbon monoxide. Of particular importance for drug metabolism are CYP2C9, CYP2C19, CYP2D6 and CYP3A4, whereas CYP1A1, CYP1A2, CYP1B1, CYP2E1 and CYP3A4 are the most important isoforms responsible for metabolic activation of precarcinogens. The continuous generation of new variants of the cytochrome P450 genes and the genetic drift has created a global situation with large interethnic differences in the distribution of the polymorphic forms of P450 genes (Kalra, 2007).

6. CYP1A1:

CYP1A1 (cytochrome P450, subfamily 1A, polypeptide 1) encodes the enzyme aryl hydrocarbon hydroxylase (AHH), which is involved in the metabolism of PAH and estrogens. Decreased exposure to oestrogen over a lifetime is associated with a reduced risk of breast cancer and conversely, an increased exposure is associated with an elevated risk of breast cancer. Therefore, changes in the activity of AHH leading to differing levels of estrogen could affect breast cancer risk. AHH catalyses the mono-oxygenation of PAH

found in cigarette smoke to phenolic products, which may be mutagenic and carcinogenic. PAHs do cause mammary tumours in mice, and are associated with a high rate of DNA adduct formation. At present, how the two aspects of AHH activity, decreasing active estrogens and increasing carcinogen levels, affect breast cancer risk has not been resolved. Therefore, polymorphisms in *CYP1A1*, which in theory change enzymatic activity, have been studied to see whether they are associated with breast cancer risk (Weber and Nathanson, 2000; Zhang, *et al*., 2004; Boyapati, *et al*., 2005)

6.1. CYP1A1 substrates:

6.1.1. Estrogen:

Estrogen is believed to be a causal factor in postmenopausal breast cancer (Clemons and Goss, 2001) and in premenopausal breast cancer (Arslan, 2009). The biological studies in animals have clearly identified Estradiol as a carcinogen (Nandi, *et al*., 1995). Catechol metabolites of estrogen may be causal as well. Estradiol and estrone are metabolized via two major pathways, i.e by hydroxylation either at the aromatic A ring or at the D ring. Hydroxylation of the Estradiol at the A ring leads to the formation of catechol estrogens (CE), namely 2- or 4- hydroxyestrons or –estradiols while hydroxylation of estrone at the D ring yields 16α-hydroxyestrone. These catechol estrogens (CEs) are the major metabolites of estrogen (Lippert, 2000). Most oestrogen metabolites undergo an additional degradation step by conjugation, either by glucuronidation, sulfation, or O-methylation by COMT. Further biotransformation of CEs forms quinones which can, in turn, form either stable or depurinating DNA adducts, resulting in carcinogens. In humans, hepatic 2-hydroxylation is mainly catalyzed by CYP1A2, 3A3 and 3A4, while CYP1A1 is responsible for extrahepatic

2-hydroxylation (Figure 3). In contrast, CYP1B1 appears to be the main CYP responsible for the 4-hydroxylation (Figure 3) and CYP2A1 and CYP3A4 for the 16α- hydroxylation in many extrahepatic tissues such as human breast and uterus (Mitrunen and Hirvonen, 2003). The 16α -hydroxylation leads to metabolites with strong estrogenic properties that have been linked to estrogen-induced carcinogenesis in both laboratory animals (Liehr, 2000) and humans (Kabat, *et al.*, 1997). While the 2-hydroxylation products (2- OH estradiol catechol and 2-OH estrone catechol) are devoid of estrogenic activities and the 2-methoxy derivatives shown to possess anti-proliferative and antiangiogenic properties (Fotsis, *et al.*, 1994), a recent study by Arslan, 2009 suggests that shift toward 2-hydroxyestrone and particularly an elevated 2-hydroxyestrone:16α-hydroxyestrone ratio may be associated with an increased risk for estrogen receptor–positive premenopausal breast cancer. Thus, factors favoring the production of 2-OH metabolites (CYP1A1) have been studied in relation to breast cancer risk.

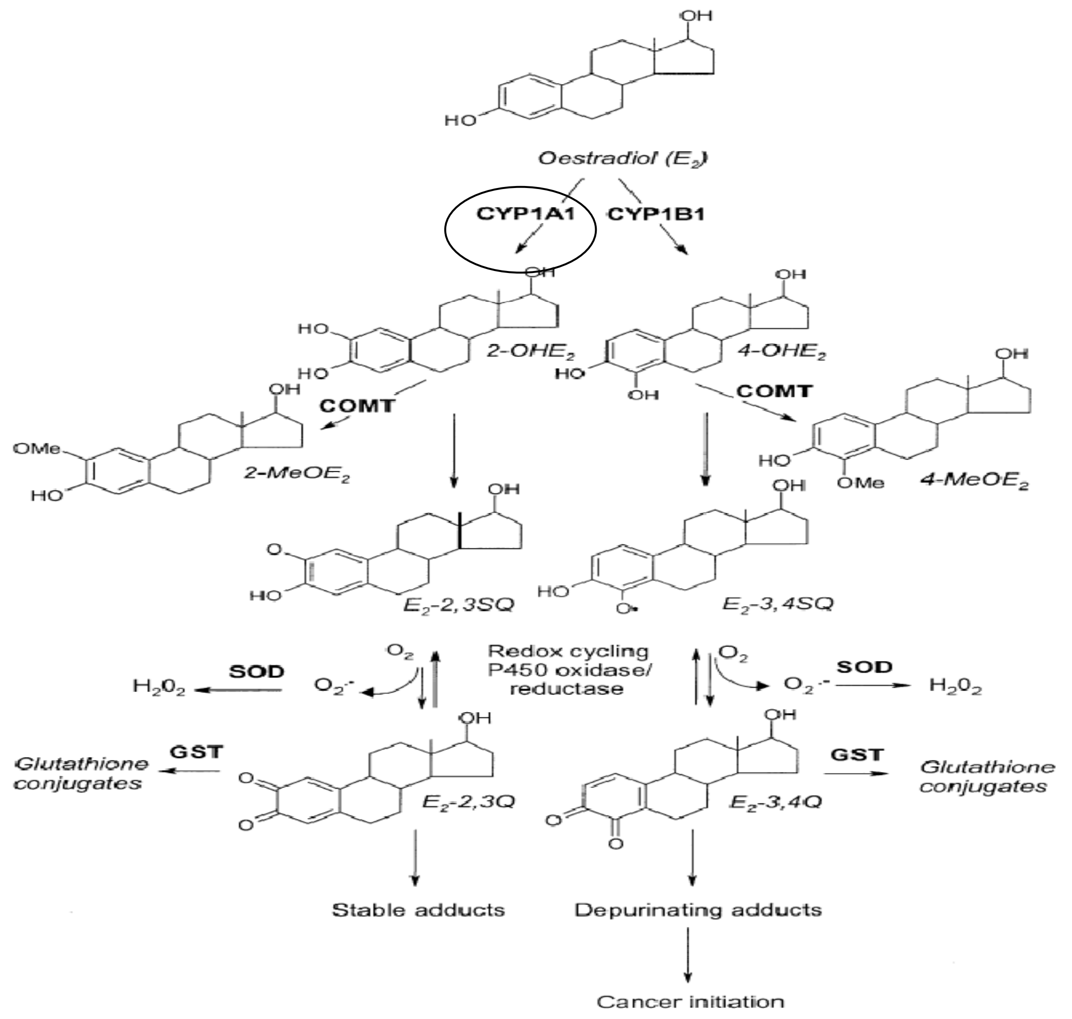


Figure 3: Metabolism of estrogen (Mitrunen and Hirvonen, 2003):
The estrogen metabolism pathway is regulated by oxidizing phase I and conjugating phase II enzymes. *CYP1A1* and *CYP*1B1 catalyze the oxidation of Estradiol (E_2) to catechol estrogens 2-OHE$_2$ and 4-OHE$_2$. The catechol estrogens are either methylated by COMT to methoxyestrogens (2-MeOE$_2$, and 4-MeOE$_2$) or further oxidized by CYPs to semiquinones (E_2-2,3-SQ and E_2-3,4-SQ) and quinones (E_2-2,3-Q and E_2-3,4-Q). The estrogen quinones are conjugated by GST to GSH-conjugates.

6.1.2. PAHs:

The last century of industrial development caused a significant increase of PAH concentrations in the natural environment. Humans encounter PAHs and HAAs from a wide range of sources such as tobacco smoke, automobile exhaust, smoked and cooked

food (ingested benzo [α] pyrene), and industrial processes. Lipophilic, these molecules are stored in the adipose tissue such as the breast. PAH are known human carcinogens and have been found to cause mammary tumours in rodents (Cavalieri N, *et al.*, 1988). Oxygenation of carcinogenic PAH and HAA (precarcinogens) by CYP1A1 gives rise to electrophilic reactive species (ultimate carcinogens) (Figure 4) that form DNA and protein adducts, leading to tumor formation and toxicity (Ma and Lu, 2007). Polymorphic variation in CYP1A1 activity could therefore modify breast cancer susceptibility.

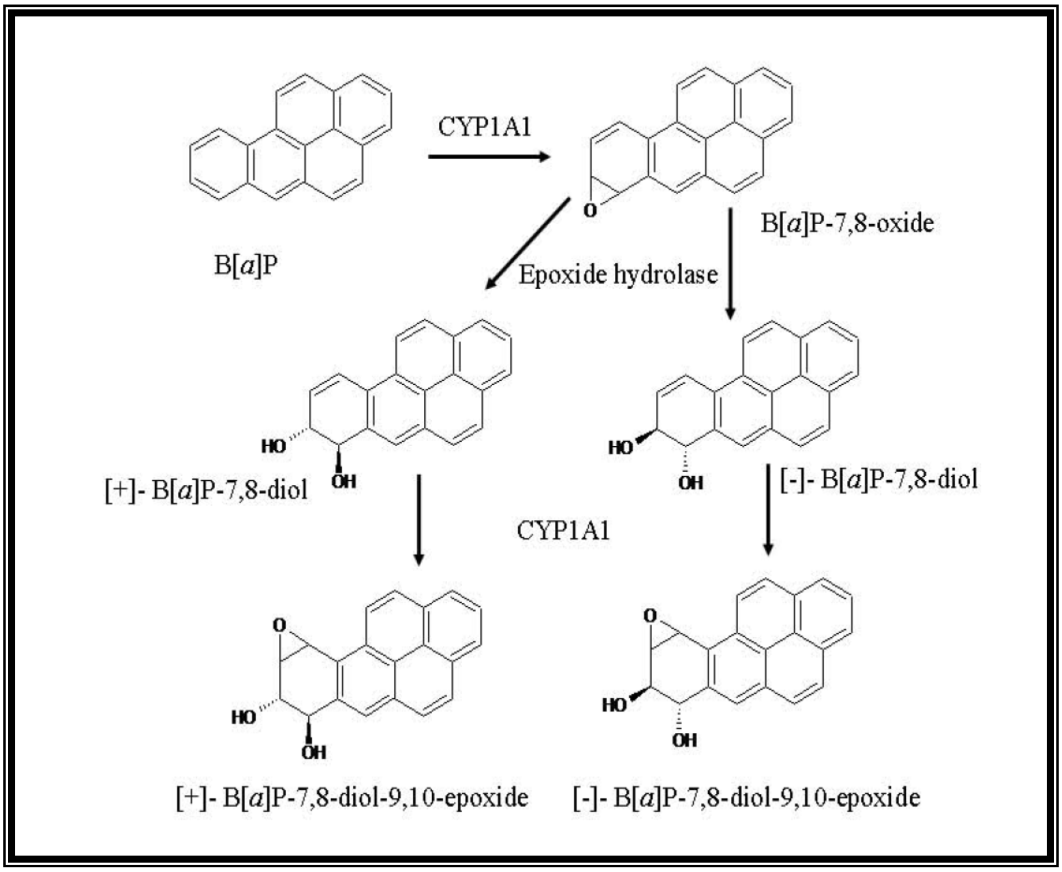


Figure 4: Metabolic activation of Benzo [α] pyrene B[*a*]P to the carcinogenic metabolites B[*a*]P-7,8-diol, B[*a*]P-7,8-diol-9,10-epoxide by CYP1A1 (modified from Androutsopoulos, *et al.*, 2009)

6.2. CYP1A1 Inducers:

*CYP*1As are highly inducible by PAHs such as benzo[a]pyrene and halogenated aromatic hydrocarbons via aryl hydrocarbon receptor (AhR)-mediated gene transcription (Bartsch, *et al.*, 2000) (Figure 5).

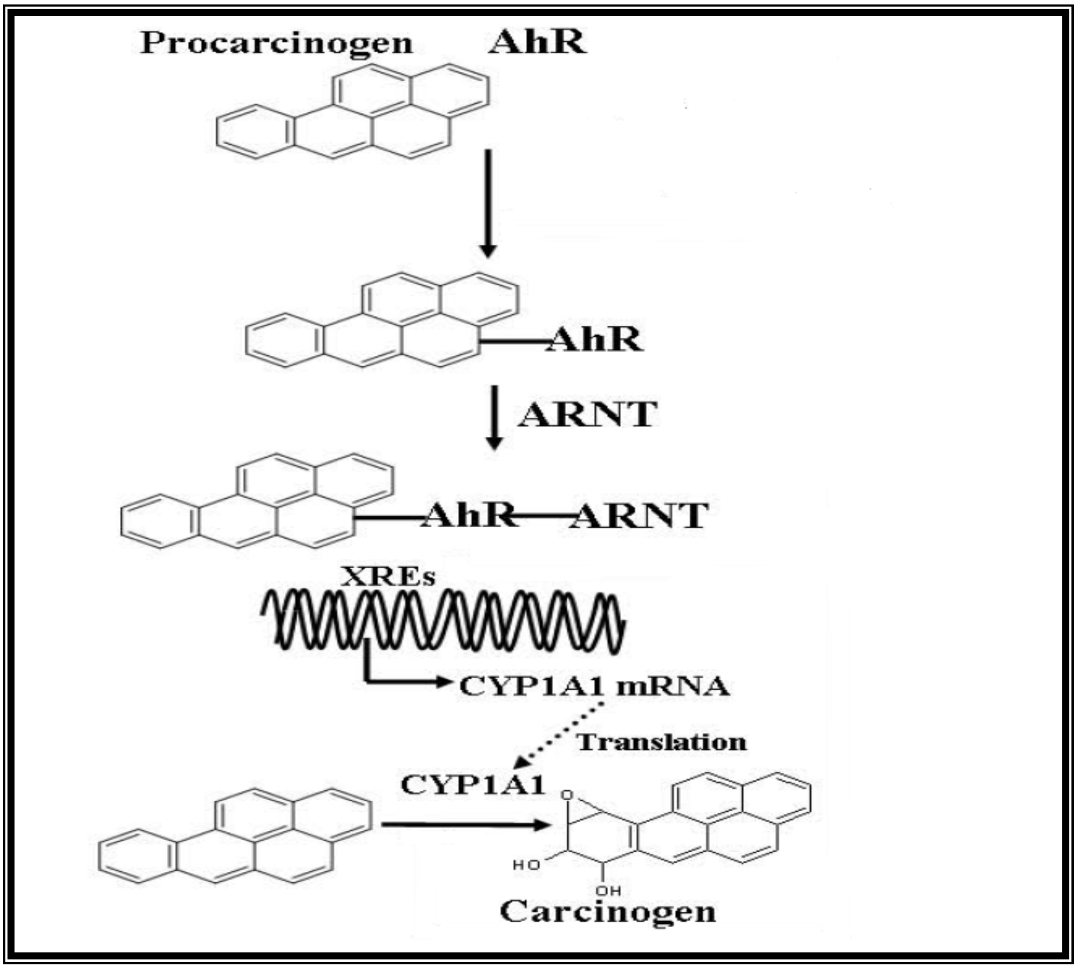


Figure 5: Mechanism of induction of CYP1A1 through the ligand (e.g. benzo[*a*]pyrene) binding to the AhR then the binding of the AhR-ARNT-benzo[*a*]pyrene complex to xenobiotic response elements (*CYP1A1*), and the formation of *CYP1A1*-mediated carcinogenic reactive intermediates, notably benzo[a]pyrene-7,8-diol-9,10-epoxide **(modified from Androutsopoulos, *et al.*, 2009)**

6.3. *CYP1A1* gene:

The *CYP1A1* gene, located on chromosome 15q24.1 (Figure 6), is 5,995 base pairs long, comprises **seven** exons and **six** intons and encodes a 512–amino acid protein (GeneCards, 2011). *CYP1A1* is highly polymorphic in humans and ethnic differences in the distribution of these polymorphisms have been reported.

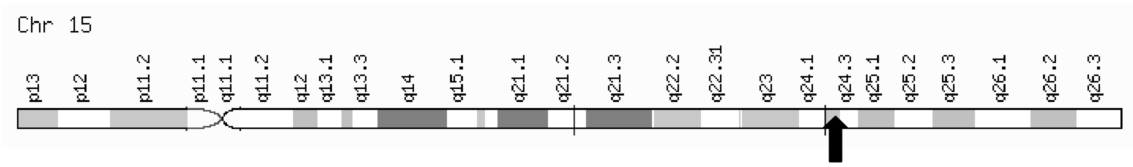


Figure 6: Schematic presentation of *CYP1A1* gene locus on chromosome 15 (Gene-cards, 2011)

6.3.1. Genetic polymorphisms of *CYP1A1*:

Several mutations have been identified in *CYP1A1* gene and mainly four polymorphisms (Figure 7) were studied in relation to breast cancer

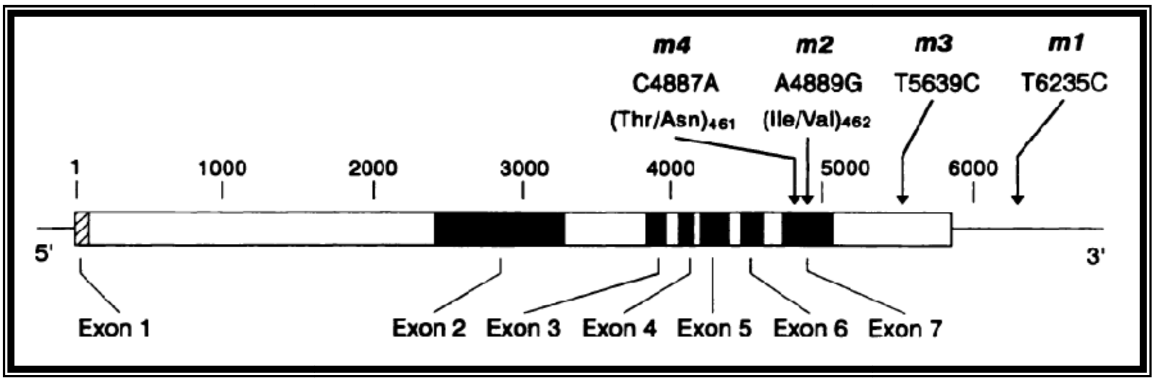


Figure 7: Localization of currently known mutations of the human *CYP1A1* gene (Cascorbi, *et al.*, 1996)

Four polymorphisms (Table 3); *M1*, *M2*, *M3*, *M4*, are currently known, the distribution of each one varies according to ethnic group: Caucasian, African and Asian. The *M1* polymorphism is located in position 6235 down-stream of the exon 7, in the 3'-flanking

region. The region is non-coding where a thymine (T) is replaced by cytosine (C) in this position a site of restriction of *Msp*1 appears. The *M2* polymorphism is the replacement in position 4889, on the exon 7, of an adenine (A) by a guanine (G). This leads to the replacement of isoleucine (Ile) by valine (Val) on amino acid 462, in the region of the heme of the P450 cytochrome. The polymorphism *M3* is the creation of a new site of restriction *Msp*1 in the intron 7. *M3* is specific of African origin ethnic groups. The polymorphism M4 has been described recently. It is located in position 4887, adjacent to the *M2* polymorphism. It corresponds to the change of a cytosine into adenine which leads to the loss of restriction site BsaI and is translated in the protein sequence by a replacement of a threonine by asparagine on the codon 461 (Surekha, *et al.*, 2009).

Significant ethnic differences have been observed in the distribution of the *CYP1A1* polymorphic genotypes (Cosma, *et al.*, 1993) with the *M1* and *M2* polymorphisms being much more frequent in Asians compared to Caucasians and African-Americans (Table 4).

Table 3: Most common *CYP1A1* SNPs (Surekha, *et al.*, 2009**)**

Mutation trivial name	**Proposed alleles nomenclature**		**Reference SNP ID**	**Nucleotide change**	**Amino-acid change**	**Location**
	Garte, 1998	**Cascorbi, *et al.*, 1996**				
M1	*CYP1A1**2	*CYP1A1**2A	rs4646903	6235T>C	None	3'-UTR
M2	***CYP1A1**3**	***CYP1A1**2C or 2B**	**rs1048943**	**4889A>G**	**462 Ile>Val**	Exon 7
M3	*CYP1A1**4	*CYP1A1**3	rs4986883	5639 T>C	None	Intron 7
M4		*CYP1A1** 4	rs1799814	4887C>A	461 Thr>Asp	Exon 7

Table 4: Frequencies of *CYP1A1* variant alleles among different populations and ethnicities

CYP1A1 polymorphism	Population (ethnicity)	Variant allele Frequency	Reference
M2 (*A4889G*)	American (Caucasian)	**10.35 %**	Ambrosone, *et al*., 1995
	American (Caucasian)	**4.13 %**	Bailey, *et al*., 1998
	Australian (Caucasian)	**3.82 %**	Hefler, *et al*., 2004
	Finish (Caucasian)	**6.51 %**	Sillanpää, *et al*., 2007
	North Indian (Asian)	**30 %**	Surekha, *et al*., 2009
	African American	**25%**	Le Marchand, *et al*., 2005
M1 (*T6235C*)	American (Caucasian)	**9.84%**	Bailey, *et al*., 1998
	Chinese (Asian)	**39 %**	Hunag, *et al*., 1999
	Chinese (Asian)	**23.7%**	Boyapati, *et al*., 2005
	American (African American)	**24.58%**	Bailey, *et al*., 1998
M3 (T5639C)	American (African American specific)	**12.02%**	Bailey, *et al*., 1998
M4 (C4887A)	Anglo-Saxon (White Caucasian)	**4.63%**	Basham, *et al*., 2001
	American (Caucasian)	**4.32%**	Bailey, *et al*., 1998

7. ***CYP1A1* polymorphism *(M2)*:**

In the present study, we evaluated the association between the *M2* polymorphism in *CYP1A1* and breast cancer risk. The *M2* polymorphism is a mutation in the heme-binding region of exon 7 of the *CYP1A1* gene at the position 4889 due to transition of the adenine by guanine (A→G), resulting in the amino-acid replacement of isoleucine with valine at the codon 462 (Ile462Val) (Surekha, *et al.*, 2009). It is conceivable that a change in the protein sequence could result in a change of the enzyme activity. The functional significance of the different *CYP1A1* genotypes in human lymphocytes has been extensively studied, but with somewhat inconclusive results (Cosma, *et al.*, 1993; Crofts, *et al.,* 1994; Landi, *et al.*, 1994; Kiyohara, *et al.*, 1996). One study indicated that participants with the variant *CYP1A1* 462Val allele had an increased inducibility of *CYP1A1* mRNA and 3-fold increase in enzymatic activity (Crofts, *et al.,* 1994). Another showed that the *M2* polymorphism alters enzyme kinetics in generating diol metabolites from benzo[α]pyrene (Schwarz, *et al.*, 2001). Similarly, lymphocyte *CYP1A1* enzyme activity was significantly increased among participants with the variant *CYP1A1* 462Val allele according to two other studies (Cosma, *et al.*, 1993; Kiyohara, *et al.*, 1996), whereas other studies did not find any change in enzyme kinetics (Persson, *et al.*, 1997) or B[α] P activation (Zhang, *et al.*, 1996). In contrast, the variant *CYP1A1* 6235C allele has been reported to be more readily inducible or to have higher activity than the wild type in human lymphocytes (Landi, *et al.*, 1994; Kiyohara, *et al.*, 1996) while in one study the T6235C polymorphism had no effect on the induction of *CYP1A1* (Crofts, *et al.,* 1994).

8. *M2* polymorphism in *CYP1A1* and breast cancer risk:

Literature review:

Potential roles of *CYP1A1* polymorphisms have been postulated for several cancers: prostate (Shaik, *et al.*, 2009), esophageal (Zhuo, *et al.*, 2009), oral and pharyngeal (Varela-Lema, *et al.*, 2008) and lung cancer (Shi, *et al.*, 2008).
On the other hand, the epidemiologic studies investigating the association between *CYP1A1* gene polymorphisms and breast carcinoma among various populations and ethnic groups have been conflicting with inconclusive results: while some have shown strong association with breast carcinoma risk, others have indicated no association.

The first study to examine the association of the *M2* polymorphism in *CYP1A1* was conducted by Rebbeck, *et al.* (1994), where 96 postmenopausal Caucasian female breast cancer patients, and 146 cancer free controls were included. No association has been reported by the researchers. Another study by Ambrosone, *et al.* (1995), recruiting 216 postmenopausal Caucasian women with incident breast cancer and 282 community controls in Western New York state to evaluate the association between *CYP1A1 M1* and *M2* polymorphisms and breast cancer risk, found the isoleucine to valine (*Ile462Val*) polymorphism in *CYP1A1* weakly, and non-significantly associated with increased breast cancer risk. However, the association between cigarette smoking, *CYP1A1* genotype, and breast cancer risk suggests that the polymorphism is associated with increased risk among lighter smokers only (<29 pack-years). In a case-control study (Shin A, *et al.,* 2007), where 513 breast cancer patients and 447 controls were recruited to evaluate the association between *CYP1A1 M1* and *M2* polymorphisms and breast cancer risk in Korean women, no association was found between the *M2* polymorphism and the breast cancer risk. However

women with *M1* mutation showed a 1.76-fold higher risk of breast cancer (95% CI= 1.19 – 2.61). On the other hand, Chacko P, *et al.* (2005) in a study on 112 South Indian female breast cancer cases and 79 controls, pointed to *CYP1A1 M1* and *M2* homozygous variants as higher susceptibility genotypes in the breast cancer patients studied (Val/Val: OR = 4.9; 95% CI: 1.3–18.4, $p = 0.017$ and Ile/Val: OR= 2.4; 95% CI= 1.3-4.6, $P = 0.007$). When stratified by menopausal status, a similar increase for both polymorphisms (*M1* and *M2*) was observed, but only in premenopausal women (OR= 3.7; 95% CI = 1.5-9.1).
A case-control study in Chinese population composed of 104 women with histologically confirmed breast cancer and 154 noncancerous controls, investigated the association between serum Organochlorines pesticides (DDTs) level, *CYP1A1* genetic polymorphism and risk of breast cancer, and found that *CYP1A1 M2* genetic polymorphism was associated with a non-significant increased risk of female breast cancer (OR = 2.61, 95% confidence interval (CI): 1.00 - 6.80) while DDT exposure might have increased the risk of breast cancer among premenopausal women with *CYP1A1 M2* variant genotype (OR = 4.35, 95% CI: 1.140 - 16.950) (Li, *et al.*, 2006).

A different result was seen in a case-control study (Singh N *et al.*, 2007) conducted in North Indian population involving 105 female breast cancer patients and 116 normal healthy female controls. This study revealed a protective association of *M2* (Ile462Val) polymorphism of *CYP1A1* gene with breast cancer development in postmenopausal women (OR=0.33; 95% CI: 0.12-0.89; *P*-value 0.03). These results represent a replication of the previous findings in a study conducted on 195 cases and 272 controls in Japan in which the variant allele 462Val carriers also showed a significantly ($p < 0.05$) reduced risk (odds ratio 0.66; 95% CI 0.45-0.96) as compared with non-carriers (Miyoshi, *et al.,* 2002). Also, a

population-based case-control study (Boyapati, *et al.*, 2005) in Chinese women involving 1134 patients with breast carcinoma and 1227 controls found that combined homozygosity for *M1* and *M2* alleles may be associated with reduced breast cancer risk, particularly among postmenopausal lean women (OR =0.52; 95% CI = 0.28-0.94) with long-term endogenous estrogen exposure (OR= 0.43; 95% CI = 0.19-0.99).

Several studies in the period up to October 2009 have been summarized in the meta-analysis by Sergentanis and Economopoulos (2009) that has examined the association between the four polymorphisms in *CYP1A1* and breast cancer risk and pointed to *A4889G* (*M2*) G allele as a risk factor for breast cancer among homozygous Caucasian participants, while it confirmed the null associations concerning the remaining three polymorphisms.

II. AIMS AND SPECIFIC OBJECTIVES:

1. The main aim of this work was to study the association between *M2* polymorphism of the low-penetrance gene *CYP1A1* and susceptibility to breast cancer in Jordanian women.
2. A secondary objective of this study is to assess the association between *M2* polymorphism in *CYP1A1* and breast cancer risk in subgroups stratified by menopausal status, histopathological type and hormone receptor status.
3. Assess the potential interaction of *M2* polymorphism with other breast cancer risk factors (smoking, environmental factors, OCs use, … etc)

III. Methodology

1. Materials:

- **Wizard® Genomic DNA Purification Kit** (Promega, USA) contains the following solutions: Cell Lysis solution, Nuclei lysis solution, Protein precipitation solution, DNA rehydration solution.
- **Absolute Ethanol 96%** (Ginaland Chemical Company, UK).
- **Isopropanol** (BioBasic Inc, Canada).
- **NucleoSpin® Blood DNA purification Kit** (Macherey Nagel, Germany) supplied with the following contents: Lyophilized proteinase K, Proteinase buffer, Buffer B3, Buffer B5, Buffer BW, Buffer BE, Nucleospin® Blood columns, 2 ml collecting tubes.
- **GoTaq® DNA Polymerase** (Promega, USA) containing 5u/µl Taq polymerase, supplied with 5X Green GoTaq® Reaction Buffer and 5X Colorless GoTaq® Reaction Buffer (Both buffers contain $MgCl_2$ at a concentration of 7.5mM for a final concentration of 1.5mM in the 1x reaction).
- **PCR Nucleotide Mix** (Promega, USA): A premixed solution containing sodium salts of dATP, dCTP, dGTP and dTTP, each at a concentration of 10mM in water; the final concentration of each dNTP in a total volume of 50µl PCR reaction is 200µM.
- ***CYP 1A1*, *M2* Primers** (e-oligos, Gene Link™, USA):
- **$MgCl_2$,** molecular biology grade (Promega, USA): supplied as 25mM $MgCl_2$ solution.
- **Nuclease free water (NFW)** (Promega, USA).

- ***BsrDI* restriction enzyme** (New England Biolabs, USA)
- **Seakem ® LE Agarose,** molecular biology grade (Lonza, USA).
- **Accugene ® TBE** (Tris-Borate-EDTA) **Buffer**; molecular biology grade (Lonza, USA).
- **Ethidium Bromide;** molecular biology grade (Electron, England).
- **50 bp DNA Ladder** (Fermentas, EU).
- **15 ml centrifuge tubes** (Jet Biofil®, China)
- **1.5 ml microcentrifuge tubes.**
- **0.2 µl thin wall PCR tubes with flat caps** (Bio-Rad, USA)

2. Equipments:

- **PTC- 100 Peltier Thermal Cycler** (MJ Research, USA).
- **Sigma 1-14 microcentrifuge** (Sigma, Germany).
- **Rotina 380 R Centrifuge** (Hettic Zentrifugen, Germany).
- **TS-100 Thermo shaker** (Biosan, Latvia)
- **Gel Doc- It™ Imaging system** (UVP, UK)
- **Electrophoresis power supply** (Apelex, France)
- **Electrophoresis Chamber or Tank** (Cleaver, USA).
- **Smart Spec™ Plus spectrophotometer** (Bio-Rad, USA)
- **Microwave** (Panasonic, USA).
- **Balance** (Denver Instrument Company, USA)
- **AF – 10 Distillizer** (Elga, UK)

- **Auto-clave** (Vision scientific Co.LTD, Korea).
- **0.5 – 10 micropipette** (VWR, Pennsylvania, USA)
- **2 – 20 µl micropipette (** VWR, Pennsylvania, USA)
- **20 – 200 µl micropipette** (Genex-Beta, UK).
- **100 – 1000 micropipette** (Genex-Beta, UK).

3. Ethical approval and Informed consent process:

The study was approved by Local Research Ethics Committee at Jordan University Hospital (JUH) (appendix I), and King Hussein Cancer Center (KHCC) (Appendix II). Verbal approval was obtained from the Jordan Hospital Ethics committee. Written informed Consents (Appendix III and IV, and V) were obtained from all participants.

4. Study and Participants Profile:

This case-control study included 112 female breast cancer patients, and 115 age- matched controls with no previous or present cancer history and who have undergone mammography with or without breast ultrasound and had negative results. All samples were collected in the period from July 2010 to February 2011 in three major hospitals in Amman: Jordan University Hospital, King Hussein Cancer Center and Jordan Hospital.

4.1. The Breast Cancer Patients Group:

One hundred and twelve consecutive breast cancer female patients that were on their active follow-up were recruited from two hospitals in Amman: 94 cases at the Hematology / Oncology and the Breast Surgery Clinics at JUH, and 18 cases at the Breast Surgery Clinic at KHCC.

4.1.1. Inclusion Criteria:

- Jordanian breast cancer patients, including newly diagnosed cases, cases diagnosed years back and the recurrent cases.

4.1.2. Exclusion Criteria:

- Non- Jordanian breast cancer patients.

4.2. The Control group:

One hundred and fifteen female controls have been recruited from three different hospitals in Amman: 35 females have been recruited during the National Screening Program at Jordan Hospital, 37 and 43 women have been recruited from the Breast Imaging Units at the JUH and KHCC, respectively.

4.2.1. Inclusion Criteria:

- Age-matched women (age-matching was performed in decades) with no history of any malignant disease and who underwent mammography with or without breast ultrasound and had negative results (BI-RADS I, BIRADS II: including benign macrocalcification, vascular calcifications, scattered microcalcifications and benign cysts).

4.2.2. Exclusion Criteria:

- Non- Jordanian females.

- Females whose mammogram showed benign conditions that may increase the risk of breast cancer notably fibrocystic changes such fibroadenoma.

- Women who underwent a prior breast biopsy.

5. Sample size calculation:

Sample size was calculated according to the following equation:

$$N = \frac{P * Q * t^2}{d^2}$$

Where:

N = sample size

t= t-statistic for the desired level of confidence, and can be obtained from statistical tables which contain the area under the normal curve (in the current study a confidence interval of 95% was chosen).

P= estimate of expected proportion of the variable of interest in the population (In the current study, P represents the prevalence or the frequency of the variant allele or *G* allele).

The frequency of *G* allele in Caucasian women ranged from 4% (Bailey, *et al*., 1998) to 12% (Ambrosone, *et al*., 1995)

Q= 1-P

d = desired level of precision (the difference that is considered sufficient). According to Ambrosone, *et al*., 1995, the difference observed in the frequency of *G* allele was about 5% (12.5% in patient group and 8 % in control group).

So by replacing: P by 8% (intermediate value), d by 0.05 (5% acceptable difference) and t by 1.96 for a confidence interval of 95%, we obtained the following sample size

$$N= \frac{0.08*(1-0.08)*1.96^2}{0.05^2} = \text{ 113 participant in each arm}$$

6. Data collection:

During a face to face interview conducted by a trained Clinical Pharmacy Master Student, a structured questionnaire was used to obtain detailed information on demographic factors, menstrual, reproductive and breastfeeding history, hormone use, age at diagnosis, dietary habits, previous malignant diseases history, physical activity, tobacco and alcohol use, and

family history of breast cancer. Most of the participants had their waist and hip circumferences measured. The body mass index (BMI) was also recorded. Clinical history including the size of the tumour, presence of axillary lymph nodes, extent of metastasis (stage), histopathological type of the tumour, hormone receptor and HER-2 status was obtained from the patient file.

This data collection form was designed by the study researchers based on the data on risk factors of breast cancer reported in the literature. A part of this data collection form is a modification of a questionnaire previously designed by Elyyan, R (MSc of Public Health) (Elyyan and Al-Jaghbeer, 2005).

Appendix VI contains data collection form for breast cancer patients and Appendix VII contains data collection form for controls (the abbreviated version of Appendix VI).

7. Samples collection:

After having the consent form signed, 3 ml of peripheral blood were collected in an EDTA anti-coagulant-containing vacutainer tube under sterile conditions. Samples were stored at 4ºC until DNA extraction was performed. Some samples (20 samples) have been stored at -20ºC if the extraction was not performed in the next 7 days.

8. Genomic DNA extraction:

DNA was extracted using two methods

8.1. Solution- Based DNA extraction:

This method was used for fresh blood samples or samples stored at 4ºC for maximum up to one week.

1. Centrifugation of a whole blood sample was performed to separate the material into three layers: a red bottom layer comprised mainly of red blood cells, a top plasma layer and a thin white layer at the interface that is enriched for white blood cells.
2. Once separated, a Pasteur pipette has been used to carefully collect the enriched white cells (buffy coat) from the interface (a small volume of plasma and RBCs has been taken with the buffy coat). Typically, this leads to a tenfold concentration of the white cells in a blood sample (e.g., a 3ml Vacutainer® tube will yield a 0.3ml buffy coat sample).
3. Genomic DNA specimens for cases and controls were extracted from buffy coat fractions using Promega Wizard kit,
   - 3.1 Up to 0.5 ml of blood containing 0.3ml of buffy coat and extra-volume of plasma and RBCs has been transferred to a centrifuge tube (15ml Dnase/Rnase free) for DNA isolation.
   - 3.2 Three-fold volume of cell lysis solution (1.5 ml) was added to 0.5 ml of buffy coat. The tube was inverted 5–6 times to mix. The mixture was then incubated for 10 minutes at room temperature (inverted 2–3 times once during the incubation) to lyse the red blood cells and centrifuged at 4,000× g for 2-5 min at room temperature.

3.3 Supernatant was discarded without disturbing the visible white pellet (WBCs)

3.4 The WBCs were resuspended by vortex for 10-15 seconds.

3.5 Nuclei Lysis solution (300µl) was added to the tube containing the resuspended cells. The solution was then pipetted 5-6 times to lyse the WBCs until the solution became very viscous.

3.6 Protein precipitation solution (100µl) was added to the nuclear lysate and vortexing was done for 10-20 seconds.

3.7 The mixture was centrifuged at 4000 rpm for 3 minutes at room temperature. A dark brown pellet should be visible.

3.8 The supernatant was transferred to a sterile 1.5 microcentrifuge tube containing 300µl of isopropanol.

3.9 The solution was then gently mixed by inversion until the white thread-like strands of DNA formed a visible mass.

3.10 The solution was centrifuged at 13,000 rpm for 5 min at room temperature. The DNA was visible as a small white pellet.

3.11 The supernatant was decanted, 300 µl of 70% ethanol was added to the DNA. The tube was gently inverted several times to wash the DNA pellet and sides of microcentrifuge tube, and then centrifuged as in step 3.10.

3.12 The ethanol was aspirated carefully using a sequencing pipette tip. The tube was then inverted on a clean absorbent paper and the pellet air-dried for 10 minutes.

3.13 Once dry, rehydration solution (volume depends on the pellet size, 100–150 µl) was added to the tube and DNA rehydrated by incubating the solution overnight at 4ºC.

3.14 Finally DNA was stored at -20ºC.

8.2. Column- based DNA extraction:

Another easy, less time consuming and more suitable extraction kit from (MACHEREY-NAGEL, Germany) was used for frozen samples (almost 20 samples). We used this method in case of failure to extract DNA from the frozen blood samples by the above mentioned solution-based kit which gave poor yield because of blood coagulation. The column-based method used in this study contains Proteinase K enzyme, a serine proteinase that has high potency in digesting and removing proteins from blood samples, thus giving better yield with frozen samples.

According to the kit manufacturer recommendation, the following procedure was followed:

- Into a 1.5 ml microcentrifuge tube, 25µl proteinase K and 200 µl lysis buffer B3 were added to 200 µl of whole blood. The mixture was mixed vigorously by vortex, and then incubated at 70 ºC for 30 min until the lysate became brownish.
- 200 µl of ethanol (96%) was added.
- The mixture was transferred to the Nucleo-spin® column in a 2 ml collection tube and centrifuged for 1 min at 11,000 x *g*. The collecting tube with flow-trough was discarded.
- The column was placed into a new 2 ml collecting tube and 500 µl pre-wash buffer BW was then added and centrifuged at 11,000 x *g* for 1 min. The collecting tube with flow-through was then discarded.
- After transferring the Nucleo-spin® column to a new collecting tube, 600 µl washing buffer was added and centrifuged at 11,000 x *g* for 1 min.

- In order to dry the silica membrane, the column was placed into a new collecting tube and centrifuged again at 11,000 x *g* for 1 min. During this step, residual ethanol is removed.
- The column was placed into a 1.5 ml microcentrifuge tube, and 100 µl pre-warmed elution buffer BE (70 ºC) was added and incubated at room temperature for 1 min. The eluted DNA is obtained by centrifugation at 11,000 x *g* for 1 min.
- The DNA samples were stored at -20 ºC.

8.3. Determination of DNA Concentration:

DNA concentrations were determined by spectrophotometric measurement of the amount of UV irradiation absorbed by the bases.

8.3.1. Preparation DNA samples

In a separate sterile 1.5ml microcentrifuge tube (for each sample), 4 µl of DNA was mixed with 96µl of NFW (this represents a 1:25 dilution of the DNA samples). The mix was then vortexed and the solution incubated for 10 minutes to ensure the complete diffusion of DNA throughout the solution.

8.3.2. Preparing the spectrophotometer:

- The spectrophotometer was first turned on and left for 10 minutes to warm up the UV light.

- On the spectrophotometer “DNA/RNA” button was pressed, then by using the “Select” button, double strand DNA “dsDNA” was selected. These settings were validated by pressing “Enter”.
- The display then asking if the conversion factor (50µg/ml) is correct, to answer, the button “Enter” was pressed. The assay was so ready for absorbance readings.
- To account the DNA concentration in the original stock, a “dilution factor” was selected and the value 25 entered and validated by pressing “Enter”.

8.3.3. Samples reading:

- To start the quantification, the spectrophotometer was blanked by inserting 100 µl of NFW in the quartz cuvette and “Read Blank” button pressed.
- To begin the reading of the DNA sample, the “>” button was pressed.
- The cuvette was removed, the blanking water discarded and replaced by the 100µl of the diluted DNA solution. The “Read sample” button was then pressed.
- After 3 seconds, an absorbance reading appeared on the screen.
- The absorbance at 260 nm **“A_{260}”** is the amount of light absorbed by the DNA. This value was used by the device to provide the concentration of DNA in each sample (A_{260} of 1 = 50 µg/ml DNA). Absorbance readings of 0.5– 1.0 were considered valid.
- **“Conc”**: represents the concentration of DNA in the original sample given according to the conversion factor and dilution factor set up in the beginning.

DNA Concentration = A_{260} (nm) x 50 (µg/ml*nm) x dilution factor (25)

- A concentration of > 50 µg/ml is considered sufficient for PCR.

- **A_{260} / A_{280}**: this ratio gives an estimate of DNA purity (contaminants in the sample, such as proteins, absorb at a wavelength of 280nm). Pure DNA has an A_{260}/A_{280} ratio of 1.7-1.9.
- To read other samples, the cuvette was removed and washed several times with NFW water, then carefully all traces of liquid were removes. Another diluted sample was added to the cuvette and the previous steps were repeated (no need to re-blank the spectrophotometer again).

8.4. Genotyping analysis :

For determination of *M2* (*A4489G*) polymorphism in *CYP1A1* gene, a 204-bp DNA fragment was amplified and restricted using the Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) based assay.

M2 mutation is an A to G nucleotide substitution at position 4889 which results in amino acid change of isoleucine to valine at codon 462 within the heme binding domain of exon 7.

8.4.1. Primers preparation:

Primers for amplification of *CYP1A1 A4889G* region were obtained from Gene-Link^{TM} Company (USA) as dried lyophilized powder. The final concentration required for PCR reaction was 0.4 μM of each primer.

- Forward Primer powder (51800 pmol) was reconstituted in a volume of 518 μl of Nuclease Free Water (NFW) to obtain a stock solution of forward primer with a concentration of approximately 100μM. Then, 20 μl of this stock solution was

diluted with 80µl of NFW to obtain a master solution with the concentration of 20µM for PCR reaction.

- Reverse Primer (49200 pmol) was prepared as described above except that the powder was reconstituted in 492 µl of NFW.
- Forward and Reverse primers sequences and melting temperature are summarized in Table 5:

Table 5: Sequence and characterization of *CYP1A1 A4889G* primers:

	Sequences	TM	P. From	P. To
F	5'- CTGTCTCCCTCTGGTTACAGGAAG-3'	63.9ºC	45803669	45803692
R	5'-TTCCACCCGTTGCAGCAGGATAGCC-3'	65.5ºC	45803489	45803513

F: forward primer, R: reverse primer, TM: melting temperature, P: position of primers according to NT_010194.17 accession number.

8.4.2. PCR Mix preparation:

Target DNA was amplified in a 50 µl of PCR mixture containing the ingredients showed in Table 6:

Table 6: PCR mixture components and the corresponding volumes and final concentrations:

Component	Final Volume	Final Concentration
DNA template	2µl	(300-500ng)
Taq polymerase	0.3 µl	1.5 U
dNTP Mix, 10mM each	1µl	0.2mM each dNTP
5 x Taq polymerase green buffer	10 µl	1x (containing 1.5mM $MgCl_2$)
Reverse primer	1 µl	0.4 µM
Forward primer	1 µl	0.4 µM
$MgCl_2$ (25 µM)	2 µl	1 mM
NFW	32.7 µl	None.

8.4.3. PCR conditions:

After optimization, the PCR reaction was performed under the following conditions:

1. Initial Denaturation: at 94°C for 5 minutes.
2. 40cycles of:
    - Denaturation: at 94 °C for 30 seconds.
    - Annealing: at 55 °C for 1 min.
    - Extension: at 72 ºC for 30 seconds.
3. Final extension: at 72º C for 5 minutes.

8.5. Digestion of PCR product with BsrDI (RFLP) :

The PCR product (204 bp) with the wild-type allele was digested with the enzyme **BsrDI** (New England Biolabs) that works at 65°C overnight to 2 fragments (149 bp and 55 bp), whereas the PCR product with the *CYP1A1* 4889G* allele remained undigested (204 bp).

Recognition Site of the enzyme:

5′...GCAATGNN▼...3′
3′...CGTTAC▲NN...5′

Where N= any base (A, C, G, T).

The digestion mix was prepared as following:

1. 20 µl PCR product
2. 2.5 µl Restriction enzyme buffer (10 X)
3. 0.8 µl (1.6 Units) restriction enzyme.
4. 1.5µl Nuclease Free Water.
5. 0.25 µl Bovine Serum Albumin "BSA" (100X).

Samples that show heterozygote or homozygote variant pattern were repeated to confirm the results.

8.6. Gel electrophoresis of the PCR and digested products:

The 2.5 % and 4% agarose gels were used to detect the PCR product of 204 bp and the corresponding RFLP products. A small 8x10cm gel (minigel) was prepared as follow

- 2.5 g (or 4g) of Agarose powder were weighed into a 250 µl conical flask.
- 100 µl of TBE buffer (1X) was added and swirled to mix.
- The mix was microwaved for about 1 minute to dissolve the Agarose powder.

- The mix was then left to cool on a bench for 1 minute.
- Under a fume hood, 20 μl of Ethidium bromide was added and swirled to mix. (The reason for allowing the agarose to cool a little before this step is to minimise production of ethidium bromide vapour).
- The gel was then poured slowly into the tray. It was left to set and solidify for at least 20 minutes and later lodged into the gel tank.
- 1 x TBE buffer was poured into the gel tank to submerge the gel.
- The first well of the gel was loaded with the Marker (ladder).
- The samples were loaded in the rest of wells.
- The gel tank was closed, the power-source switched on, and the gel was run at 130 volts.
- The progress of the gel was monitored by reference to the dye.
- After a sufficient progress, the gel tank was switched off and unplugged.
- The gel was then carried to a UV light box (UV-trans-illuminator) and a picture of the fluorescent ethidium bromide-stained PCR product was taken with the Gel Doc-It™ Imaging system.
- The expected results of PCR-RFLP are shown in Figures 8 and 9:

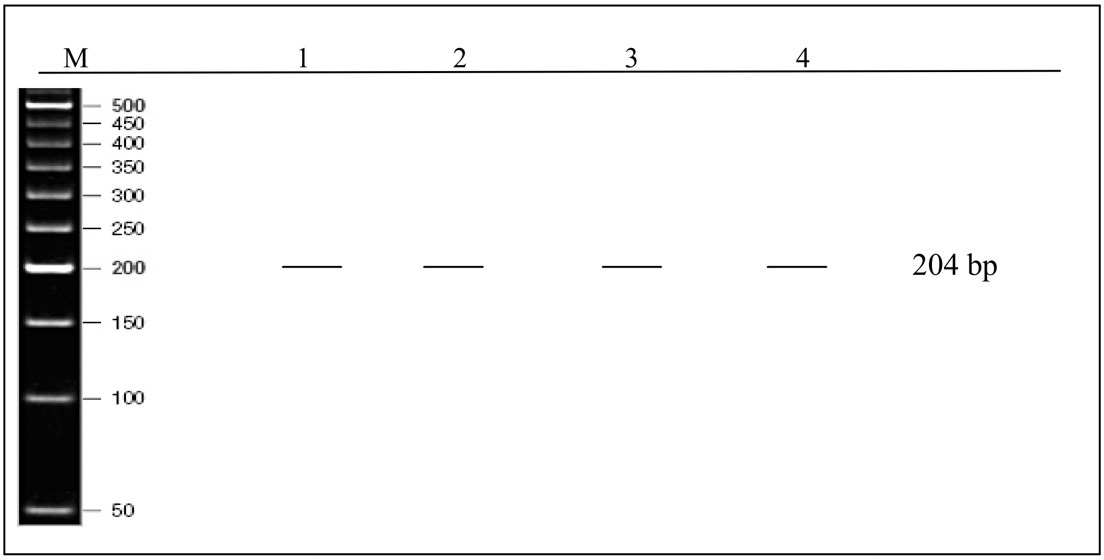


Figure 8: Schematic presentation of expected PCR result for the *CYP1A1 A4889G* polymorphism: Lane M: 50 bp DNA step Ladder. Lanes 1-4: PCR products

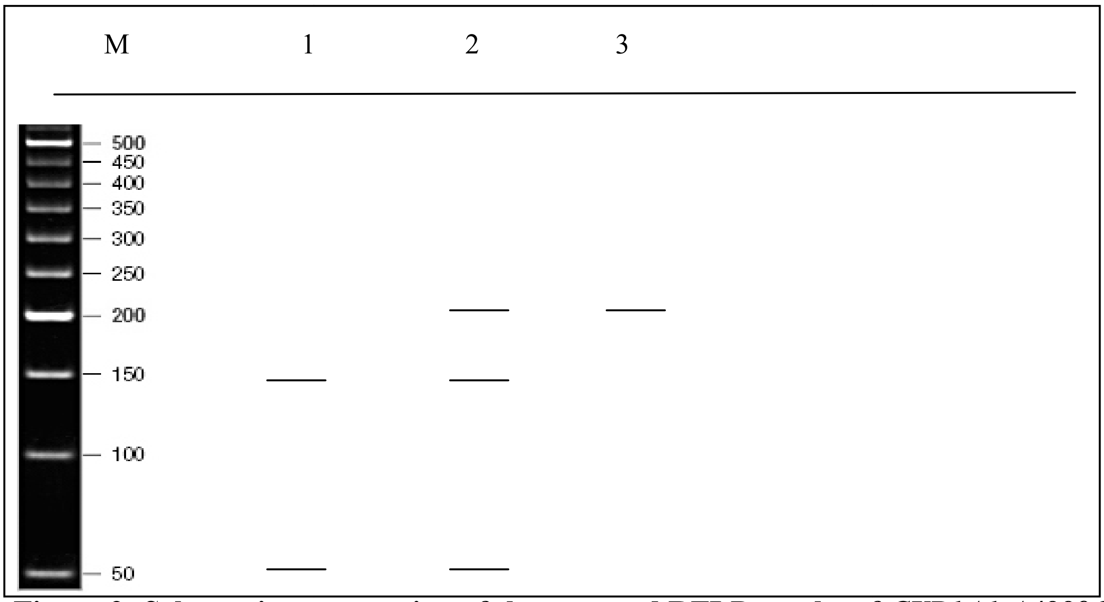


Figure 9: Schematic presentation of the expected RFLP results of *CYP1A1 A4889G* polymorphism: Lane M: 50bp DNA step ladder, Lane 1: wild type AA (149 and 55bp); Lane 2: heterozygote variant AG (204, 149 and 55bp); Lane 3: homozygote variant GG (204 bp).

9. Estimation of genotype and allele frequencies:

Genotype frequencies for *CYP1A1 A4889G* allele among female Jordanian population was estimated from the results of PCR-RFLP test for 112 female breast cancer patients and 115 female controls. This estimation was according to the following formulas (Brooker, 2005)

$$\text{Allele frequency} = \frac{\text{Number of copies of an allele in a population}}{\text{Total number of all alleles for that gene in a population}}$$

$$\text{Genotype frequency} = \frac{\text{Number of individuals with a particular genotype in a population}}{\text{Total number of all individuals in a population}}$$

Also, the genotype and the allele frequencies were compared with Hadry-Weinburg Equilibrium (the calculations are illustrated in Appendix VIII).

10. Statistical analysis:

The association between *CYP1A1 M2* polymorphism and breast cancer risk was analyzed by calculating the crude odds ratio (OR) and 95% confidence interval (95% CI) using Chi-square test. Individual demographic and clinical factors were also examined for their relations to breast cancer using Chi-square test for categorical variables **(**educational level, family monthly income, age at menarche, period regularity, hormone replacement therapy use, oral contraceptive use, parity, breast-feeding, cigarette smoking, age at first cigarette

smoking, number of pack-years, physical activity, duration of physical activity, family history of breast cancer in first degree relatives, physical trauma to the breast, alcohol use and dietary fat intake). The independent-sample t-test was used for the continuous variables (age, BMI, WHR, age at menopause, duration of HRT use, number of full- term pregnancies, age at FFTP, total duration of breast feeding, duration of oral contraceptive use). We also combined *G* homozygotes with the *A/G* heterozygotes in these analyses. Subgroup analysis based on menopausal status and smoking history was carried out separately for premenopausal and postmenopausal women and for the ever smokers and never smokers, respectively, to explore the potential modifying effect of non-genetic factors in the *CYP1A1 A4889G*- breast carcinoma association.

Women were considered to be postmenopausal if they had reported natural menopause determined as cessation of menstruation for at least for 12 months, or had undergone bilateral oophorectomy. Women who were hysterectomized with intact ovaries (ovary) or for whom details of the operations were unknown were classified as postmenopausal if they were no longer menstruating and were older than 51 years or had used hormone replacement therapy. All the rest were classified as premenopausal (Sillanpää, *et al.,* 2007). In breast cancer patients criteria for menopausal status were applied to the age of diagnosis rather than to the age at the encounter, i.e., if the patient was still menstruating at the time of diagnosis, she was considered to be pre-menopausal. The patient was considered to be post-menopausal if she had experienced menopause before the date of diagnosis, and the cessation of menstruation has been for one year or more. Age at menopause was set to age at which menstrual periods ended or age of first use of hormone therapy, whichever came first.

IV. RESULTS:

A sample of Gel Electrophoresis results is shown in Figures 11 and 12.

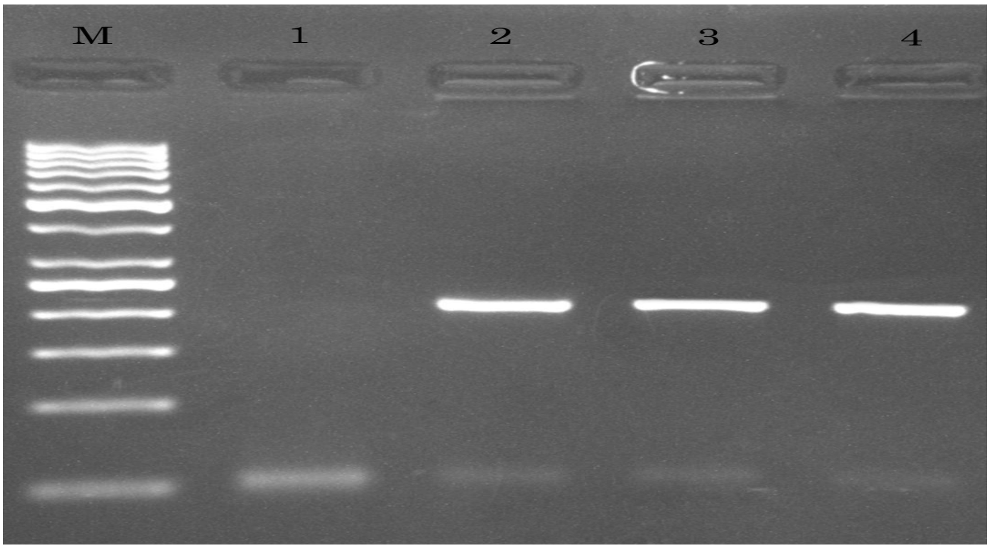


Figure 10: 2.5% Agarose gel electrophoresis (1xTBE) of undigested PCR product for 3 samples. Lane M: 50 bp DNA step ladder. Lane 1: negative control. Lanes 2, 3 & 4: PCR product (204 bp).

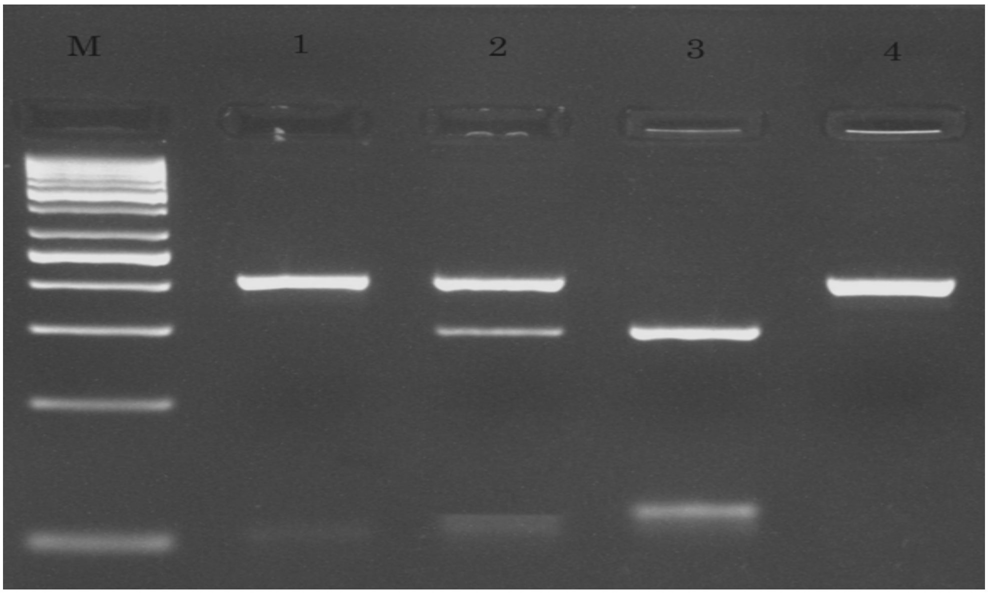


Figure 11: 4% Agarose gel electrophoresis (1x TBE) of the RFLP products. Lane M**:** 50 bp step ladder. Lane 1: undigested PCR product .Lanes 2,3 & 4: *BsrDI* digested RFLP products. Lane 2: Heterozygote variant type AG (204, 149 & 55bp). Lane 3: homozygote wild type AA (149 & 55 bp). Lane 4: homozygote variant GG (204bp).

1. Characteristics of study participants:

In the current study, the genotypes of *CYP1A1 M2 (A4889G or Ile462Val)* were studied in 112 Jordanian female breast cancer patients and 115 age-matched Jordanian female controls who have undergone mammography with or without breast ultrasound and have been shown to be breast cancer free. All participants included in this study were of the same phenotypic ethnic group (Caucasians). The response rate was almost 90% among breast cancer patients and 85% among Controls.

The demographic characteristics and the putative risk factors of breast cancer investigated in this study are listed in Tables 7, 8, 9, 10 and11.

As shown in Table 7, the age of breast cancer patients did not statistically differ from controls (p= 0.69) (Mean age = 50.18 ± 9.43 years vs. 48.03 ± 8.21 years respectively). No significant difference was observed between cases and controls in terms of reproductive factors (Table 8), exogenous hormone intake (Table 9), smoking history, alcohol and dietary fat intake (Table 10). However, controls had a higher family income (p=0.004), a higher educational level (p= 0.009) (Table 7) and tended to be more physically active (p= 0.001) than patients (Table 10). An interesting observation, women with breast carcinoma frequently reported direct hit (physical trauma) to the breast (p= 0.001) (Table 11). Thirty percent of patients and 32% of controls were postmenopausal (p= 0.88). (Table 8)

Table 7: Demographic characteristics of study participants:

Characteristics	Cases (n=112)* N (%) or mean ± SD	Controls (n=115)* N (%) or mean ± SD	*P* value: breast carcinoma cases vs. controls
Age	50.18 ± 9.43	48.03 ± 8.21	0.69
Educational level			
• Illiterate / elementary school	21 (18.7%)	7 (6.1%)	**0.009**
• Middle and High school	31 (27.7%)	44 (38.3%)	
• College or higher	60 (53.6%)	64 (55.6%)	
Family monthly income			
• <250JD	26 (25 %)	12 (10.4%)	
• >250JD	78 (75%)	103 (89.6%)	**0.004**

***:** Numbers of participants vary due to missing information.

Table 8: Reproductive characteristics of study participants:

Characteristics	Cases (n=112)* N (%) or mean ± SD	Controls (n=115)* N (%) or mean ± SD	*P* value: breast carcinoma cases vs. controls
Age at menarche			
• <14	61 (55.4%)	76 (66.6%)	
• ≥14	49 (44.6%)	38 (33.4%)	0.76
Regular period			
• Yes	92 (83 %)	100 (87%)	
• No	19 (17%)	15 (13%)	0.39
Regular period			
• Yes	92 (83 %)	100 (87%)	
• No	19 (17%)	15 (13%)	0.39
Menopausal status			
• Pre-menopausal	78 (69.64%)	79 (68.70%)	
• Post-menopausal	34 (30.36)	36 (31.30%)	0.88
Age at menopause	44.44± 9.07	46.50 ± 7.29	0.30

Parity • Nulliparous • Parous	21 (18.7%) 91 (81.3%)	13 (11.3%) 102 (88.7%)	0.12
Number of full-term pregnancies	4.11 ± 2.03	4.15 ± 2.11	0.88
Age at first delivery	24.55 ± 5.42	23.35 ± 5.14	0.12
Breastfeeding • Yes • No	87 (77.7%) 25 (22.3%)	96 (83.4%) 19 (16.6%)	0.27
Total duration of breastfeeding in years	3.67 ±4.27	3.61± 2.86	0.91

***:** Numbers of participants vary due to missing information.

Table 9: Use of female hormone medications by study participants:

Characteristics	**Cases (n=112)*** **N (%) or mean ± SD**	**Controls (n=115)*** **N (%) or mean ± SD**	***P* value: breast carcinoma cases vs. controls**
HRT [a]: • Ever users • Never users	6 (17.7%) 28 (82.3%)	9 (25.7%) 26 (74.3%)	0.42
Years of HRT use	2.80 ± 5.04	2.86 ± 3.55	0.98
Oral contraceptives • Ever users[b] • Never users	50 (44.6%) 62 (55.4%)	57 (49.6%) 58 (50.4%)	0.458
Years of oral contraceptives use	3.87 ±4.06	2.90 ± 3.59	0.19

***:** Numbers of participants vary due to missing information.
a: hormone replacement therapy.
b: ever users are defined as women who used oral contraceptives for a duration equal or longer than 1 month.

Table 10: Lifestyle characteristics of the study participants:

Characteristics	Cases (n=112)* N (%) or mean ± SD	Controls (n=115)* N (%) or mean ± SD	*P* value: breast carcinoma cases vs. controls
BMI [a]	28.65 ± 5.41	27.7 ± 4.34	0.15
WHR [b]	0.87 ± 0.07	0.85 ± 0.10	0.07
Cigarette Smoking			
• Ever smokers	18 (16%)	29 (25%)	
• Never smokers	94 (84%)	86 (75%)	0.09
Age at first cigarette smoking			
• <20 years old	3 (16.6%)	5 (17.2%)	1.00
• >20 years old	15 (83.4%)	24 (82.8%)	
Duration of smoking (years)	18.22 ± 6.12	14.11± 9.21	0.10
Average number of pack-years [c]	11.94 ± 7.62	9.52 ± 8.87	0.34
Physical activity in the last 5 years [d]			
• Yes	45 (40.1%)	72 (62.6%)	**0.001**
• No	67 (59.9%)	43 (37.4%)	
Duration of physical activity			
• <2hours./week	10 (22.2%)	8 (11.2%)	
• 2-4 hours/week	17 (37.8%)	32 (44.4%)	0.27
• ≥5hours/week	18 (40%)	32 (44.4%)	
Alcohol intake:			
• **Regular drinker**	0 (0%)	0 (0%)	
• **Occasional drinker**	7 (6.3%)	13 (11.3%)	0.179
• **Never drinker**	105 (93.7%)	102 (88.7%)	
High fat intake			
• **Yes**	32 (28.6%)	25 (21.7%)	0.23
• **No**	80 (71.4%)	90 (78.3%)	

***:** Numbers of participants vary due to missing information.
a: Body mass index, **b**: waist to hip circumference ratio, **c**: the pack-years is a way to measure the amount a person has smoked over a long period of time. It is calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked: Number of pack years = (number of cigarettes smoked per day x number of years smoked)/20 (1 pack has 20 cigarettes). **d**: Physical activity in the last 5 years for controls and the 5 years prior to diagnosis for patients.

Table 11: Miscellaneous breast cancer risk factors:

Characteristics	**Cases (n=112)*** **N (%) or mean ± SD**	**Controls (n=115)*** **N (%) or mean ± SD**	***P* value: breast carcinoma cases vs. controls**
Family history of breast cancer in the first degree relatives			
• Yes	16 (14.3%)	10 (8.7%)	0.19
• No	96 (85.7%)	105 (91.3%)	
Direct hit to the breast			
• Yes	20 (17.85%)	10 (8.7%)	**0.001**
• No	92 (82.15%)	105 (91.3%)	

*****:** Numbers of participants vary due to missing information.

All breast cancer patients had pathologically confirmed breast carcinoma. The histopathological features and hormone receptors and/or HER-2 expression by their tumours are summarized in Table 12. Mean age at diagnosis among patients was 46.19 (± 9.81) years. The majority of the tumours were presented on the right breast (55.4%), were invasive of ductal histology (75%), diagnosed at advanced stages (stage III- IV =54%), and were estrogen and progesterone receptor positive (60.7%), and HER-2 negative (44.6%)

Table 12: Histopathological Characteristics of Breast Carcinoma Patients:

Clinical Parameter	No. of Patients (%)*
Breast involved	
• Left	41 (36.6%)
• Right	62 (55.4%)
• Bilateral	9 (8%)
Tumour stage	
• Stage 0	3 (2.7%)
• Stage I	13 (11.6%)
• Stage IIA	28 (25%)
• Stage IIB	9 (8%)
• Stage IIIA	21 (18.8%)
• Stage IIIB	10 (8.9%)
• Stage IIIC	5 (4.5%)
• Stage IV	18 (16.1%)
• Missing	5 (4.5%)
Histopathological type	
• Invasive Ductal Carcinoma	84 (75%)
• Invasive Lobular Carcinoma	11 (9.8%)
• Ductal Carcinoma in situ	5 (4.5%)
• Lobular Carcinoma in situ	1 (0.9%)
• Others	7 (6.3%)
• Missing	4 (3.6%)
Hormone receptor expression	
• ER[a]+/PR[b]-	14 (12.5%)
• ER+/PR+	68 (60.7%)
• ER-/PR-	2 (1.8%)
• ER-/PR+	19 (17%)
• Missing	9 (8.0%)
HER-2 expression:	
• HER-2[c] (+)	42 (37.5%)
• HER-2 (-)	50 (44.6%)
• Missing	20 (17.8%)

a: Estrogen Receptor
b: Progesterone Receptor
c: Human Epidermal growth factor Receptor 2

2. Genotyping and allelic frequencies of *CYP1A1 A4889G*:

Among 112 breast cancer patients 102 (91.07%) were homozygote wild type (AA), 10 (8.93%) were heterozygote variant type (AG) and none (0%) was homozygote variant type (GG). Of the 115 controls, 102 (88.70%) were homozygote wild type (AA), 12 (10.43%) were heterozygote variant type (AG) and only 1 (0.87%) was homozygote variant type (GG) (Table 13).

Table 13: Frequency of *A4889G* polymorphism among 112 breast cancer patients and 115 age-matched controls.

Genotype	Number of patients (%)	Number of controls (%)
4889AA	102 **(91.07%)**	102 **(88.70%)**
4889AG	10 **(8.93%)**	12 **(10.43%)**
4889GG	0 **(0%)**	1**(0.87%)**
Allele frequency		
***A* allele**	214 **(95.53 %)**	216 **(93.92%)**
***G* allele**	10 **(4.46 %)**	14 **(6.08 %)**

The genotype frequencies were consistent with expectations from Hardy-Weinberg equilibrium in both: patients (χ^2= 0.10; *p* =0.75) and control groups (χ^2= 0.087; p=0.77). (Table 14)

Table 14: Comparisons of genotyping results in the present study with Hadry-Weinberg (H-W) expectations*:

Genotype	Patients	H-W expectations	*p*-value/ χ^2	Controls	H-W expectations	*p*-value/ χ^2
AA	102	102	1.00 /	102	101	0.78/
AG	10	10	0.00	12	13	0.087
GG	0	0	(<3.14)	1	0	(<3.14)

*H-W calculations are shown in appendix VIII

3. Association between *CYP1A1 M2* polymorphism and breast cancer :

Because of the extremely low frequency of the *GG* genotype among the study participants, we combined the variant heterozygote *AG* and variant homozygote variant *GG* groups into the same group of *G* allele carriers. Compared to controls, patients carrying at least one *G* allele were not significantly different (*p*- value = 0.55; OR = 0.77; 95% CI= 0.32 – 1.83). (Table 15)

Table 15: Association between *CYP1A1 A4889G (M2)* genotypes and the breast cancer risk:

Genotype	Patients	Controls	OR (95% CI)
4889 AA	102 (91.70%)	102 (88.4%)	
4889 AG + GG	10 (8.93 %)	13 (11.6%)	**0.77 (0.32 – 1.83)**

4. Further analysis:

No significant association was found between the *CYP1A1 A4889G* genotypes and breast cancer risk in subgroups defined by menopausal status (Table 16). Similarly, neither smoking history (Table 17) nor oral contraceptives (OCs) use (Table 18) had an impact on the relation of *CYP1A1 A4889G* genotype to breast cancer.

Table 16: Association between *CYP1A1 A4889G* genotypes and breast cancer according to menopausal status

Genotype	**Total Sample** Patients/ Controls	**Premenopausal** Patients / Controls	**Postmenopausal** Patients / Controls
AA	102 / 102	69 / 68	33 / 34
AG + GG	10 / 13	9 / 11	1 / 2
***p*- value**	0.55	0.65	0.59
OR (95%CI)	**0.77 (0.32 – 1.83)**	**0.8 (0.31 – 2.06)**	**0.51 (0.04 – 5.96)**

Table 17: Association between *CYP1A1 A4889G* genotypes and breast cancer according to smoking status:

Genotype	**Total Sample** Patients/ Controls	**Ever Smokers** Patients / Controls	**Never smokers** Patients / Controls
AA	102 / 102	18 / 25	84 / 77
AG + GG	10 / 13	0 /4	10 / 9
***p*-value**	0.55	0.28	0.97
OR (95%CI)	**0.77 (0.32 – 1.83)**	**0**	**1.09 (0.39 – 2.64)**

Table 18: Association between CYP1A1 A4889G genotypes and breast cancer according to OCs use

Genotype	**Total Sample** Patients/ Controls	**Ever used OCs** Patients / Controls	**Never used OCs** Patients / Controls
AA	102 / 102	45/ 51	57 / 51
AG + GG	10 / 13	5 / 6	5 / 7
***p*- value**	0.55	0.92	0.46
OR (95%CI)	**0.77 (0.32 – 1.83)**	**0.94 (0.27 – 3.3)**	**0.64 (0.19 – 2.14)**

Since the proportion of women using HRT was low (5.35% among patients and 7.83% among controls), we were unable to properly assess the potential impact of this factor on the association between *CYP1A1 A4889G* polymorphisms and breast cancer in our study population.

Also, because of the low frequency of *G* allele-containing genotypes among study participants, we were unable to conduct a further analysis of the association between these genotypes and histopathological parameters.

We also conducted a subgroup analysis (Table 19) based on menopausal status to investigate the association between breast cancer and the lifestyle factors (such as BMI and WHR) which showed different influences on postmenopausal as compared to premenopausal breast cancer in some previous studies.

Table 19: Association between lifestyle factors and breast cancer risk

Risk factor	**Premenopausal women**		***p*- value**	**Postmenopausal women**		***p*- value**
	Patients	**Controls**		**Patients**	**Controls**	
BMI(kg/m^2)	27.7 ± 4.8	27.3 ± 4.1	0.63	30.7 ± 6.1	28.4 ± 4.8	0.08
WHR	0.86 ± 0.07	0.85 ± 0.12	0.64	0.92 ± 0.07	0.86 ± 0.07	**0.001**

V. DISCUSSION:

This case-control study analyzed the association between breast cancer and the *M2* genetic polymorphism of the gene that encodes a carcinogen and estrogen metabolizing enzyme, CYP1A1. To the best of our knowledge, this is the first study to include extensive information on lifestyle, hormonal and environmental risk factors for breast cancer allowing a comprehensive assessment of their interaction with *M2* genetic polymorphism and breast carcinoma among Jordanian women. Furthermore, this is the first case-control study that used high accuracy in selecting control participants, as all of the women in our control group have undergone mammography with or without breast ultrasound and have been proven to be breast malignancy-free. In addition, our population of Jordanian women is relatively homogenous, as all of the study participants are classified into the same single ethnic group (Caucasian) which minimizes potential bias due to population stratification.

The response rate was almost 90% among breast cancer patients. Thus, the cases can be viewed as representing unselected typical breast cancer patients from Amman area. Similarly, response rate among controls was almost 85%. It should, however, be kept in mind that people who agree to participate in a study tend to be different from the remaining population in relation to demographic and lifestyle factors. For example, they can have a healthier way of life or be better educated (Sillanpää, *et al*., 2007).

In this study, we tested the hypothesis that *CYP1A1 M2* polymorphism at the exon 7 has an impact on the breast cancer. It has been postulated that the genotype and phenotype patterns of this cytochrome P450 enzyme may modulate the qualitative and quantitative patterns of

the products and, thus, determine the susceptibility to cancer by increasing the expression of the gene or the activity of the protein produced (CYP1A1) (Chacko, *et al*., 2005). Four polymorphisms in *CYP1A1* gene have been mainly studied in relation to breast cancer. A functional role of these polymorphisms has been essentially proposed for the *M2* polymorphism with some conflicting results. Some studies suggested an increased enzymatic activity (Cosma, *et al.*, 1993; Schwarz, *et al.*, 2001) and others showed no change in the enzyme function (Zhang, *et al.*, 1996; Persson, *et al.*, 1997).

A wide ethnic variation in the distribution of this polymorphism was observed, with the *G* variant allele being much higher in Asians (≈ 30% according to Huang, *et al*., 1999) than in Caucasians (3 % according to Hefler, *el al*., 2004; 6% according to Zhang *et al.*, 2004 and10% according to Ambrosone, *et al.*, 1995) with an intermediate value in African-Americans (21% according to Le Marchand, *et al*., 2005). In the present study frequency of the *M2* variant allele "*G* allele" among controls (6.08%) is in accordance with that of Caucasians (Table 20) and in disagreement with previous results reported among Jordanian women (38% according to Manna'a, *et al*., 2006).

Table 20: Frequencies of *A4889G* polymorphism in *CYP1A1* in different populations:

Population (sample size: cases/controls)	AA	AG	GG	A allele	G allele	Reference
Jordanian Caucasian (112/115)	102/102 **(91.07 / 88.7%)**	10 / 12 **(8.93 / 10.43%)**	0 /1 **(0 / 0.87%)**	214/ 216 **(95.53 / 93.91%)**	10/14 **(4.46 /6.08%)**	Present study
Jordanian (111/111)	31/32 **(27.9 / 28.8%)**	75/74 **(67.6 / 66.7 %)**	5/5 **(4.5 / 4.5 %)**	137/138 **(62 / 62%)**	85/84 **(38 / 38 %)**	Manna'a, *et al.*, 2006
American Caucasian (176 /228)	140 / 195 **(80/ 85 %)**	32 /31 **(18 / 14%)**	4 /2 **(2 / 1 %)**	312 / 421 **(97.5 / 98.8%)**	40 / 35 **(12.5 / 8.2%)**	Ambrosone, *et al.*, 1995
American Caucasian (164/ 162)	146/ 150 **(90.9/ 92.6 %)**	15 / 12 **(9.2%/ 7.4 %)**	0 /0 **(0 / 0 %)**	313 / 312 **(95.43 / 96.3 %)**	15 / 12 **(4.57 / 3.7 %)**	Bailey, *et al.*, 1998
Australian Caucasians (390 / 1693)	361 / 1570 **(92.6 / 92. 7%)**	28 / 117 **(7.2 / 6.9 %)**	1 / 6 **(0.4 / 0.2 %)**	750 / 3257 **(96.15 / 96. 2%)**	30 / 129 **(3.85 / 3.8 %)**	Hefler, *et al.*, 2004
Finish Caucasian (481/479)	426 / 412 **(88.6 / 86.0 %)**	53 / 66 **(11 / 13.8 %)**	2/1 **(0.4 /0.2 %)**	905 / 890 **(94.07/ 92.9 %)**	57 / 68 **(5.93/ 7.1%)**	Sillanpää, *et al.*, 2007
Chinese Asian (150 /150)	49 / 48 **(34.7/ 33. 1%)**	60 / 80 **(42.6/ 55.2%)**	32 / 17 **(22.7/11. 7%)**	158 / 176 **(56 / 61 %)**	124 /114 **(44 / 39%)**	Huang, *et al.*, 1999
North Indian Asian (248/249)	78 / 122 **(31.6 / 49%)**	169 / 127 **(68 / 51 %)**	1 / 0 **(0.4 / 0 %)**	325 / 371 **(66 / 74 %)**	171 / 127 **(34 / 26%)**	Surekha, *et al.*, 2009

Data presented as numbers and %.

CYP1A1 A4889G and breast cancer association studies have raised conflicting results. The present study showed the lack of significant overall association as well as lack of association in premenopausal or postmenopausal women being in good agreement with several other studies (Rebbeck, *et al.,* 1994; Bailey *et al.*, 1998; Basham, *et al.*, 2001; Sillanpää, *et al.,* 2007). However, reduced risk of breast cancers was observed among Asian *G* allele carriers, in whom the frequency of *4889G* allele is almost 4 fold compared to our population (> 25.4% vs. 6.08%), including Japanese women (OR=0.66; 95% CI= 0.45-0.96) (Miyoshi, *et al*., 2002), North Indian postmenopausal women (OR=0.33; 95% CI= 0.12-0.89) (Singh, *et al*., 2007), Chinese postmenopausal women who are homozygous for both *CYP1A1 M1* and *M2* variant alleles (OR= 0.43; 95 CI= 0.19 – 0.99) (Boyapati, *et al*., 2005), and, finally, Korean women only when the *M1* and *M2* variant alleles were combined together (OR = 0.59; 95% CI = 0.43- 0.80) (Shin, *et al.,* 2007). On the other hand, a significant increased risk was observed among Caucasian women (in Connecticut) with at least one *4889G* variant allele (OR= 3.6; 95% CI = 1.5 - 8.2) (Zhang, *et al*., 2004), among Asian women in North India (OR= 2.08; 95% CI= 1.45-3.03) (Surekha, *et al.*, 2009) and in South Indian premenopausal women (OR= 3.7; 95% CI= 1.5-9.1) (Chacko, *et al*., 2005). Also, a recent meta-analysis by Sergentanis and Economopoulos, 2009 found that the *G* allele increases the risk of breast cancer among homozygous Caucasian participants.

In many of the studies that failed to find any overall associations between the *CYP1A1* genotypes and breast cancer, significant associations between the *CYP1A1* polymorphisms and smoking habits (Ambrosone, *et al.*, 1995) or higher levels of serum Polychlorinated Biphenyls (PCBs) in relation to risk of breast cancer have been observed (Laden, *et al.*, 2002). However, our results are consistent with other studies that found no association

between the smoking habits, the *CYP1A1 A4889G* polymorphism and breast cancer risk in Caucasians (Krajinovic, *et al*., 2001; Li, *et al*., 2004). The proportion of women who smoke was low (16 – 25%) in our study which is in accordance with the results of the latest survey published by the Jordanian Department of Statistics (2010). PCB level has not been assessed in our study.

The discrepancies in association of *CYP1A1 M2* variant genotype and breast cancer risk in different studies may be explained, in part, by ethnic differences in the frequency of the genotypic polymorphism. Almost all studies assessing this association are based on case-control study design. Whether the *CYP1A1M2* polymorphism is significant in such a design depends on the frequency of the variant allele in the controls as well as in the cases. The significantly low prevalence of *G* allele in Caucasian populations, including our own, results in a lower statistical power for detecting an effect of this polymorphism on breast cancer risk. Our study was only powered enough to detect a difference of 3% which may explain the null association observed among this Jordanian population. In contrast, in Asian studies, more than 10% of the study population possesses the variant genotype; therefore, adequate statistical power facilitates the evaluation of such an association.

Another crucial issue is differences in environmental factors and exposure levels. The gene-environment interactions may differ between different study populations and, to be detectable, they require a relevant exposure. Therefore, data on level of relevant exposures are important in assessing the association between certain polymorphic genes and cancer. The genotyping methods used in different laboratories may also differ with respect to the sensitivity and specificity of the technique, and this may, therefore, result in different levels

of misclassification in genotype data (Brennan, 2002). In our study, genotyping results were interpreted by two independent laboratory workers and random samples were repeated to assure reproducibility.

Study of non-genetic risk factors for breast cancer among Jordanian females showed a significantly reduced risk associated with physical activity (OR = 0.4; 95% CI= 0.23 – 0.7; $p = 0.01$) and a significantly increased risk associated with physical trauma to the breast (OR = 2.28; 95% CI = 1.02 – 5.13; $\boldsymbol{p} = 0.001$). The latter being consistent with findings of another case-control by Rigby, *et al.,* (2002), in which physical trauma to the breast has been associated with a 3 fold increased risk of breast cancer (OR= 3.3; 95% CI = 1.3 - 10.8, $p < 0.0001$).

Trauma from blows may produce cicatricial masses which increase in size, restrict the skin of the breast, and are very difficult to distinguish on physical examination from carcinoma. Van Netten, *et al.,* (1994) suggested that tissue injury in the area of in-situ carcinoma of the breast could be a factor in the development of invasive cancer. The theory is that rupture of the ducts filled with in-situ carcinoma cells due to blows or severe compression could result in spillage or dislocation of these cells into the stroma. Tumor cells under these conditions would then be subjected to abnormal regulation, mimicking a continual healing process; it is known that metastases can arise at such sites (Van Netten, *et al.*, 1994). Macrophages are present in large numbers in most breast carcinomas, and interaction with the tumor cells could result in invasion and distant spread (Van Netten, *et al.,* 1993).

The relationship between physical activity and breast cancer has been reported as complex, particularly in premenopausal women. Some studies, have shown a decreased risk of premenopausal breast cancer in women who exercise more (Maruti, *et al.*, 2008), but others

have shown no difference (Mertens, *et al.*, 2006**).** In the present study, we found a 60% reduced risk in premenopausal women who exercise more (OR= 0.4; 95% CI= 0.21-0.77; p = 0.006). Actually, in premenopausal women, even moderate physical activity can be associated with anovulatory cycles, which are associated with decreased risk. Among postmenopausal women, the literature more consistently shows a protective effect of regular strenuous activity on breast cancer incidence (McTiernan, *et al.*, 2003).

Regarding other risk factors, this study showed no significant overall difference between patients and controls regarding reproductive and hormonal factors, lifestyle (BMI, WHR), and dietary factors (fat intake). However, more pronounced impact of higher WHR (OR=7.60; 95%CI = 2.31- 24.9) was observed among postmenopausal women (p= 0.001) (Table 19). The increased risk of breast cancer among obese postmenopausal women is attributed to the synthesis of estrogens in fat depots (Feigelson, *et al.,* 2004).

VI. CONCLUSIONS:

1. In Jordanian breast cancer female patients, the *CYP1A1 A4889G* genotype frequencies of the homozygous wild type (AA), heterozygous variant type (AG) and homozygous variant type (GG) are 91.07%, 8.93% and 0% respectively. Among the controls, these frequencies are 88.07%, 10.43% and 0.87% respectively. The allelic frequency of the *CYP1A1 4889G* allele is 4.46 % among breast cancer patients and 6.08% among controls.

2. There is no overall association between *CYP1A1 M2* polymorphism and breast cancer among Jordanian females, even after stratifying participants by menopausal status or smoking habits.

3. Physical trauma to the breast was associated with 2 fold increased risk of breast cancer among Jordanian females.

4. Physical activity resulted in 60%-reduced breast cancer risk among premenopausal women in our study.

5. Higher waist-to-hip circumference ratio was associated with 7 fold increased breast cancer risk only among postmenopausal women.

VII. STUDY LIMITATIONS:

1. Small sample size because of the high per subject cost of DNA technologies and time constrains, which might have resulted in inability to detect an association of a rare mutation such as the *CYP1A1 4889G* with breast cancer.

2. The low frequency of the variant allele along with the small sample size did not allow a proper subgroup analysis and a better understanding of interaction of risk factors for breast cancer with the genotype studied.

3. The reliance on self-report for some data, such as cigarette smoking, dietary fat intake, alcohol use. Such factors may have been underreported by participants in general, thereby biasing our results towards the null.

4. Since the data on risk factors were retrospectively collected, this may increase the risk of recall bias especially for events that date back to years ago as age at menarche, duration of oral contraceptives use and duration of breast feeding

VIII. RECOMMENDATIONS AND FUTURE WORK:

1. Increase the sample size by recruiting more Jordanian breast cancer patients and age- matched controls to confirm or refute our findings. Based on the results obtained in the current study, we recommend a sample size of a minimum 465 participants in each group.

2. Study other polymorphisms in *CYP1A1* gene such as *M1* and *M4*.

3. More genes known to predispose to breast cancer need to be studied and gene-environment and /or gene-gene interactions elucidated (a well-designed, large scale studies are emphasized)

4. Use more proper documentation and more proper measurement of exposures to risk factors to assess their associations with breast cancer (prospective nature of the study).

5. Use more precisely measurable intermediary risk markers, like DNA adducts, cytogenic damage, and mutations rather than the presence of cancer as endpoint.

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Appendix I

Ethical Approval from Jordan University Hospital

A-ADM0112

مستشفى الجامعة الأردنية
Jordan University Hospital

Ref. ____________ الرقم: ٢٥/٢٠١٠/IRB ل

Date: ____________ التاريخ: ٢٠١٠/٧/٢٠

الدكتوره نائلة بولاتوفا

تحية طيبة و بعد،،،

الموضوع: البحث المقدم من طالبة الماجستير إيمان عمراني بعنوان:
"العلاقة بين التباين الجيني M2 في CYP1A1 و احتمالية حدوث سرطان الثدي عند النساء الأردنيات، دراسة مقطعية رصدية"
"Association of M2 Polymorphism in CYP1A1 with Breast Carcinoma in Jordanian Women, a Cross-Sectional Observational Study."

و حيث أن البحث المذكور لا يستدعي إستخدام أدوية جديدة لغايات الدراسة أو تعديل أدوية موجودة، حيث يتضمن البحث سحب دم من قسم أمراض الدم و الأورام، قسم الجراحة و قسم الأشعة (Mammography).و حيث أن القانون النافذ بخصوص الأبحاث الدوائية مقصور على إستخدامات الأدوية (Drugs) و حيث أن الطريقة التي ستقوموا بإستخدامها هي طريقة مستخدمة ضمن العناية الروتينية. لذا فإن لجنة البحث العلمي في المستشفى تعتبر لجنة مستقلة **(Independent ethics committee)** تقوم مقام اللجنة المؤسسية للدراسات الدوائية **(IRB)** في حال بحثكم، و عليه فيعتبر بحثكم مجازاً من اللجنة المؤسسية للدراسات الدوائية **(IRB)** شريطة إستخدام نموذج الإقرار و الموافقة المرفق **(Consent Form)**.

مع الشكر و التحية،،،،

نائب رئيس اللجنة المؤسسية للدراسات الدوائية (IRB)

الأستــــاذ الدكتــور مصطفــى الشنــــاق

تلفون ٥٣٥٣٤٤٤ - فاكس ٥٣٥٣٣٨٨ - صندوق بريد ١٣٠٤٦ - عمان - الأردن
Tel. 5353444 Fax 5353388 - P.O.Box 13046 - Amman - Jordan

Appendix II

Ethical Approval from King Hussein Cancer Center

King Hussein Cancer Center | مركز الحسين للسرطان

Institutional Review Board | مجلس تقييم الأبحاث

Date: 15/December/2010

Dear Dr. Mahmoud Al-Masri and Ms. Iman Amrani,

In reference to the proposal entitled: Association of M2 Polymorphism in CYP1A1 with Breast Carcinoma in Jordanian Women: a Cross-Sectional Observational Study (Proposal #: 10 KHCC 76).

You are kindly informed that the IRB has reviewed and approved your submitted documents which include the following:

1. IRB Submission FORM B
2. English Research Proposal
3. Arabic Consent Form for Patients
4. Arabic Consent Form for Controls
5. Data Collection Form for Patients (Arabic and English)
6. Data Collection Form for Controls (Arabic and English)
7. "Estimation of Sample Size When the Variable of Interest is a Dichotomous Variable" Document

Kindly Note, that this proposal was approved by convened IRB review.

Kindly note that if the study will extend beyond one year you have to submit a renewal form and an interim update on the study. At the end of the study, you are requested to submit end of study report to IRB.

Please inform the IRB office of any publications/ abstracts that may result from this research.
On behalf of all members of KHCC IRB, I would like to wish you a very successful study.

Dr. Maysa Al-Hussaini
Chairman, Institutional Review Board.
King Hussein Cancer Center

Date: 15/December/2010

The IRB consists of members of medical and non medical background including public, lawyers, nurses and pharmacists. It is the policy of the IRB to conduct random audits on a percentage of approved projects. These audits may be conducted at any time after the project starts. In cases where the IRB considers that there may be a risk of adverse events, or where participants may be especially vulnerable, the IRB may request the principal investigator to provide an outcomes report, including information on follow up of participants.
KHCC-IRB is compliant with GCP Guidelines.

Appendix III

Consent Form for Patients from KHCC

مركز الحسين للسرطان	King Hussein Cancer Center
مجلس تقييم الأبحاث	Institutional Review Board

إقرار بالموافقة على بحث بدون فائدة مباشرة للمشاركين من مجموعة مرضى سرطان الثدي

Informed Consent for Research with No Direct Benefits to Participant (Patients)

عنوان البحث :

العلاقة بين التباين الجيني *M2 في CYP1A1* و سرطان الثدي عند النساء الأردنيات: دراسة مقطعية.

الجزء الأول – معلومات للمشارك في البحث:

أ. الغرض من البحث:

سرطان الثدي هو أكثر أنواع السرطانات انتشارا عند النساء في الأردن. قد تساعدنا هذه الدراسة في فهم أحد أسباب هذا المرض و المتعلقة بحدوث تغييرات في أحد الجينات و هو ما قد يساعدنا في إيجاد علاجات جديدة أو تشخيص المرض في مرحلة مبكرة. يهدف هذا البحث لدراسة العلاقة بين التباين الجيني M2 في CYP1A1 و احتمالية حدوث سرطان الثدي عند النساء الأردنيات

ب. وصف البحث:

تتمثل هذه الدراسة في جمع عينة دم (3مل) من مجموعتين من النساء الأردنيات:

المجموعة الأولى : تتمثل في 100 مريضة مصابة بسرطان الثدي (حاليا أو سابقا)

المجموعة الثانية: تتمثل في 100 امرأة قامت باجراء صورة الماموغرافي و كانت نتيجة هذه الأخيرة سلبية.

عند الانتهاء من تجميع كل هذه العينات سيتم استخراج الحمض النووي منها، و باستخدام تقنية مخبرية اسمها PCR-RFLP سيتم دراسة العلاقة بين الطفرات التي قد تحدث في المورثات الجينية ل CYP1A1 و احتمالية الاصابة بسرطان الثدي.اذا قمت بالموافقة على المشاركة في هذه الدراسة، سيتم سحب عينة دم من الوريد (في المختبر المخصص لذلك)، و ستقوم كذلك الطالبة ايمان عمراني باجراء مقابلة معك للحصول على معلومات مفصلة كالمعلومات الديموغرافية ، والوزن ، والطول ، و بداية دورة الحيض، انتظامها، استمراريتها والحمل ، واستخدام الهرمونات ، التاريخ المرضي، والعادات الغذائية والنشاط البدني، و التدخين، والتاريخ

العائلي لسرطان الثدي ، بالإضافة إلى التعرض للإشعاع ، والتعرض للعوامل البيئية،و قياس محيط الخصر و الورك ، و كل ذلك باستخدام نموذج خاص بهذه الدراسة.

ج. المدة المتوقعة للدراسة:

المده اللازمه لانهاء الدراسه كاملة :6 أشهر.

مدة مشاركة المريض في هذه الدراسة : ستتم مشاركتك في هذه الدراسة مرة واحدة فقط، خلال الزيارة الروتينية للعيادة.

د. عدد المشاركين بالدراسة –: 100 مريضة مصابة بسرطان الثدي و 100 امرأة غير مصابة به.

و. المخاطر والانزعاجات المحتملة: هناك خطر صغير مع عملية سحب الدم ، حيث أن العملية مشابهة لأي عملية سحب من أجل الفحوص المخبرية: بعض من الألم ، ونادرا كدمات أو التهاب في الموقع الذي تم السحب منه ، وفي بعض الأحيان ، الدوار أو الإغماء. إذا شعرت بالإغماء ، فينبغي عليك أن تعلمي الممرضة.

ز.الفوائد المرجوة :

لن تكون هناك فائدة مباشرة لك عند المشاركة في هذه الدراسة. و لكن من خلال مشاركتك ، قد تساهمين في تحسين العلاج والاكتشاف المبكر لسرطان الثدي عند النساء الأردنيات.

ط. تكاليف المشاركة:

لن تكون هناك أي تكاليف من جراء المشاركة في هذه الدراسة.

ي. المكافآت المالية:

لن تكون هناك أي مكافأات مالية لدى مشاركتك في هذه الدراسة.

ك. المشاركة الطوعية:

المشاركة في هذه الدراسة طوعيه وإذا قررت عدم المشاركة فانك لن تتعرض لأي مضايقات أو لفقدان حقك المشروع في المعالجة.

ل. : السرية

كمشارك في هذه الدراسة ستكون هويتك ومحتويات ملفك الطبّي سرية في جميع المنشورات المتعلقة بنتائج الدراسة كما سيبقي رقم سجلك الطبي سري في الحدود التي يسمح بها القانون.

ويمكن الإطلاع على ملفك الطبّي من قبل مجلس تقييم الأبحاث أو الوكيل الداعم للدراسة في حدود النظم والقوانين المطبقة بهذا الخصوص سيتم تزويدك بنسخة موقعة من هذا الإقرار.

ن.: إنهاء المشاركة:

أن قرارك بالانسحاب من الدراسة لن يؤثر على تلقيك للخدمة العلاجية المعتمدة والمتوفرة في مركــز الحســين للسرطان.

- **الأشخاص الذين يمكن الاتصال بهم:**

- **في حالة وجود أي استفسار عن الدراسة أو أسئلة محدّدة تتعلّق بهذه الدراسة وفي حالة حدوث أي مضاعفات تتعلق بالدراسة نرجو الاتصال ب أ.د مجمود المصري على هاتف رقم (المستشفى) 5300460 تحويلة رقم 1673 أو جهازخلوي رقم 0777646632**
- **في حالة وجود أي أسئلة عامة أو أسئلة تتعلّق بحقوق المشارك عليك الاتصال بمجلس تقييم الأبحاث على هاتف رقم 5300460 فرعي 1669.**

الجزء الثاني: تفويض من المشارك بالدراسة التي لا يتوقع منها فائدة مباشرة للمشارك:

اسم المشارك: ---

1. أقر بأنني قد قرأت – أو شرح لي بلغة مفهومة لدي – هذه المعلومات المتعلقة بمشاركتي في هذا البحث وأن االدكتور محمود المصري أو الطالبة ايمان عمراني أوضحا لي طبيعة وأهداف هذه الدراسة والانزعاجات, كما أقر بأنه قد أتيحت لي الفرصة لتوجيه جميع الأسئلة المتعلقة بموضوع الدراسة وتلقيت الإجابات الوافيه.
2. أفهم بأن هذه الدراسة ليس لها أي فائدة علاجية مباشرة لي ومع ذلك أتطوع بالمشاركة فيها مع علمي بالمخاطر والانزعاجات الناتجة عنها.
3. وأفهم بأن لي مطلق الحرية بسحب هذا التفويض وإنهاء مشاركتي بهذه الدراسة في أي وقت أشاء مع علمي بجميع العواقب والمخاطر المترتبة على انسحابي من الدراسة (إن وجدت). كما أفهم بأن انسحابي من هذه الدراسة لن يؤثر على حقي في تلقي العناية الطبية اللازمة والتي تمنح للمشاركين بالدراسة أو استحقها في الأحوال العادية.
4. أوافق على أن يكون هذا الإقرار كمشاركة طوعيه في هذا البحث الطبي
5. كما أؤكد بأنني قد قرأت (أو قرأ لي) هذا التفويض وأن كل المعلومات اللازمة قد تمت تعبئتها بدقة قبل توقيعي عليه.
6. أقر بأني قد استلمت نسخه موقعه من هذا الأقرار.
7. قد يقوم الباحث الرئيس بطرح بعض الأسئله عليك بعد الانتهاء من شرح الاقرار.
8. . بهذا أتطوع للمشاركة في هذه الدراسه تحت إشراف أ.د محمود المصري , والمتضمن لما يلي: العلاقة بين التباين الجيني M2 في CYP1A1 و سرطان الثدي عند النساء الأردنييات: دراسة الحالات الإفرادية المقترنة بحالات ضابطة.

توقيع المريض أو ولي الأمر: ________________التاريخ : ____________________________

الاسم ______________________________ صلةالقرابة:___________________________

توقيع الشاهد:_______________________الاسم: ______________________________

الرقم الوطني : ______________________ التاريخ : ____________________________

أقر بأنني قد شرحت للمتطوع/ أو ولي أمره المذكور أعلاه بصورة كاملة طبيعة وأهداف مشروع البحث المذكور والمتضمن عدم وجود فائدة مباشرة على المشارك. كما قد شرحت المضاعفات المحتمل حدوثها من جراء هذه الدراسة سواء كانت لأسباب معروفة أو غير معروفة. كما إنه من المفهوم لدي بأنه قد فهم طبيعة الدراسة والغرض منها والمخاطر الناتجة عنها وذلك قبل توقيعه على الموافقة بالمشاركة ، ولقد قمت بتوضيح استعدادي للإجابة على أي أسئلة متصلة بهذه الدراسة ، وقمت فعلاً بالإجابة الوافيه على جميع أسئلته المتعلقة بالدراسة.

توقيع الباحث الرئيسي: _________________________ الاسم (طباعة): د. محمود المصري.

التاريخ: ____________________

تمت مراجعة هذا النموذج والموافقة عليه من قبل مجلس تقييم الأبحاث في مركز الحسين للسرطان

Appendix IV

Consent Form for Controls from KHCC

مركز الحسين للسرطان	King Hussein Cancer Center
مجلس تقييم الأبحاث	Institutional Review Board

إقرار بالموافقة على بحث بدون فائدة مباشرة للمشاركين من المجموعة الضابطة

Informed Consent for Research with No Direct Benefits to Participant (controls)

<u>عنوان البحث :</u>

العلاقة بين التباين الجيني *M2* في *CYP1A1* و سرطان الثدي عند النساء الأردنيات:دراسة مقطعية.

الجزء الأول – معلومات للمشارك في البحث:

ب. <u>الغرض من البحث:</u>

سرطان الثدي هو أكثر أنواع السرطانات انتشارا عند النساء في الأردن. قد تساعدنا هذه الدراسة في فهم أحد أسباب هذا المرض و المتعلقة بحدوث تغييرات في أحد الجينات و هو ما قد يساعدنا في إيجاد علاجات جديدة أو تشخيص المرض في مرحلة مبكرة. يهدف هذا البحث لدراسة العلاقة بين التباين الجيني *M2* في *CYP1A1* و احتمالية حدوث سرطان الثدي عند النساء الأردنيات

<u>ب. وصف البحث:</u>

تتمثل هذه الدراسة في جمع عينة دم (3مل) من مجموعتين من النساء الأردنيات:

المجموعة الأولى : تتمثل في 100 مريضة مصابة بسرطان الثدي (حاليا أو سابقا)

المجموعة الثانية: تتمثل في 100 امرأة قامت باجراء صورة الماموغرافي و كانت نتيجة هذه الأخيرة سلبية.

عند الانتهاء من تجميع كل هذه العينات سيتم استخراج الحمض النووي منها، و باستخدام تقنية مخبرية اسمها PCR-RFLP سيتم دراسة العلاقة بين الطفرات التي قد تحدث في المورثات الجينية ل CYP1A1 و احتمالية الاصابة بسرطان الثدي.اذا قمت بالموافقة على المشاركة في هذه الدراسة، سيتم سحب عينة دم من الوريد (في المختبر المخصص لذلك)، و ستقوم كذلك الطالبة ايمان عمراني (أو الطالبة حنان بطارسة) باجراء مقابلة معك للحصول على معلومات مفصلة كالمعلومات الديموغرافية ، والوزن ، والطول ، و بداية دورة الحيض، انتظامها، استمراريتها والحمل ، واستخدام الهرمونات ، التاريخ المرضي، والعادات الغذائية والنشاط البدني، و التدخين، والتاريخ العائلي لسرطان الثدي ، بالإضافة إلى التعرض للإشعاع ، والتعرض للعوامل

البيئية،و قياس محيط الخصر و الورك ، و كل ذلك باستخدام نموذج خاص بهذه الدراسة.

ج. المدة المتوقعة للدراسة:

المده اللازمه لانهاء الدراسه كاملة :6 أشهر.

مدة مشاركة المريض في هذه الدراسة : ستتم مشاركتك في هذه الدراسة مرة واحدة فقط، خلال الزيارة الروتينية للعيادة.

د. عدد المشاركين بالدراسة –: 100 مريضة مصابة بسرطان الثدي و 100 امرأة غير مصابة به.

و. المخاطر والانزعاجات المحتملة:

هناك خطر صغير مع عملية سحب الدم ، حيث أن العملية مشابهة لأي عملية سحب من أجل الفحوص المخبرية: بعض من الألم ، ونادرا كدمات او التهاب في الموقع الذي تم السحب منه ، وفي بعض الأحيان ، الدوار أو الإغماء. إذا شعرت بالإغماء ، فينبغي عليك أن تعلمي الممرضة.

ز.الفوائد المرجوة :

لن تكون هناك فائدة مباشرة لك عند المشاركة في هذه الدراسة. و لكن من خلال مشاركتك ، قد تساهمين في تحسين العلاج والاكتشاف المبكر لسرطان الثدي عند النساء الأردنيات.

ط. تكاليف المشاركة:

لن تكون هناك أي تكاليف من جراء المشاركة في هذه الدراسة.

أ‌. المكافآت المالية:

لن تكون هناك أي مكافأات مالية لدى مشاركتك في هذه الدراسة.

ك. المشاركة الطوعية:

المشاركة في هذه الدراسة طوعيه وإذا قررت عدم المشاركة فانك لن تتعرض لأي مضايقات أو لفقدان حقك المشروع في المعالجة.

ل. السرية:

كمشارك في هذه الدراسة ستكون هويتك ومحتويات ملفك الطبّي سرية في جميع المنشورات المتعلقة بنتائج

الدراسة كما سيبقي رقم سجلك الطبي سري في الحدود التي يسمح بها القانون.

ويمكن الإطلاع على ملفك الطبّي من قبل مجلس تقييم الأبحاث أو الوكيل الداعم للدراسة في حدود النظم والقوانين المطبقة بهذا الخصوص سيتم تزويدك بنسخة موقعة من هذا الإقرار.

ن.: إنهاء المشاركة:

أن قرارك بالانسحاب من الدراسة لن يؤثر على تلقيك للخدمة العلاجية المعتمدة والمتوفرة في مركــز الحســين للسرطان.

- **الأشخاص الذين يمكن الاتصال بهم:**

- **في حالة وجود أي استفسار عن الدراسة أو أسئلة محدّدة تتعلّق بهذه الدراسة وفي حالة حدوث أي مضاعفات تتعلق بالدراسة نرجو الاتصال بالدكتورة ليلى أبو طاحون على هاتف رقم 5300460 (المستشفى) تحويلة رقم 1222 أو جهازخلوي رقم 0777646858.**
- **في حالة وجود أي أسئلة عامة أو أسئلة تتعلّق بحقوق المشارك عليك الاتصال بمجلس تقييم الأبحاث على هاتف رقم 5300460 فرعي 1669.**

الجزء الثاني: تفويض من المشارك بالدراسة التي لا يتوقع منها فائدة مباشرة للمشارك:

اسم المشارك: ---

1. **أقر بأنني قد قرأت – أو شرح لي بلغة مفهومة لدي – هذه المعلومات المتعلقة بمشاركتي في هذا البحث وأن االدكتورة ليلى أبو طاحون أو الطالبة ايمان عمراني أوضحا لي طبيعة وأهداف هذه الدراسة والانزعاجات, كما أقر بأنه قد أتيحت لي الفرصة لتوجيه جميع الأسئلة المتعلقة بموضوع الدراسة وتلقيت الإجابات الوافيه.**
2. **أفهم بأن هذه الدراسة ليس لها أي فائدة علاجية مباشرة لي ومع ذلك أتطوع بالمشاركة فيها مع علمي بالمخاطر والانزعاجات الناتجة عنها.**
3. **وأفهم بأن لي مطلق الحرية بسحب هذا التفويض وإنهاء مشاركتي بهذه الدراسة في أي وقت أشاء مع علمي بجميع العواقب والمخاطر المترتبة على انسحابي من الدراسة (إن وجدت). كما أفهم بأن انسحابي من هذه الدراسة لن يؤثر على حقي في تلقي العناية الطبية اللازمة والتي تمنح للمشاركين بالدراسة أو استحقها في الأحوال العادية.**
4. **أوافق على أن يكون هذا الإقرار كمشاركة طوعيه في هذا البحث الطبي**
5. **كما أؤكد بأنني قد قرأت (أو قرأ لي) هذا التفويض وأن كل المعلومات اللازمة قد تمت تعبئتها بدقة قبل توقيعي عليه.**
6. **أقر بأني قد استلمت نسخه موقعه من هذا الأقرار.**
7. **قد يقوم الباحث الرئيس بطرح بعض الأسئله عليك بعد الانتهاء من شرح الاقرار.**
8. **. بهذا أتطوع للمشاركة في هذه الدراسه تحت إشراف أ.د محمود المصري , والمتضمن لما يلي: العلاقة بين التباين الجيني M2 في CYP1A1 و سرطان الثدي عند النساء الأردنييات: دراسة الحالات الإفرادية المقترنة بحالات ضابطة.**

توقيع المريض أو ولي الأمر: ________________التاريخ : ______________________________

الاسم ________________________________   صلةالقرابة:_________________________

توقيع الشاهد:____________________________الاسم: ______________________________

الرقم الوطني : ___________________________   التاريخ : ___________ ________________

أقر بأنني قد شرحت للمتطوع/ أو ولي أمره المذكور أعلاه بصورة كاملة طبيعة وأهداف مشروع البحث المذكور والمتضمن عدم وجود فائدة مباشرة على المشارك. كما قد شرحت المضاعفات المحتمل حدوثها من جراء هذه الدراسة سواء كانت لأسباب معروفة أو غير معروفة. كما إنه من المفهوم لدي بأنه قد فهم طبيعة الدراسة والغرض منها والمخاطر الناتجة عنها وذلك قبل توقيعه على الموافقة بالمشاركة ، ولقد قمت بتوضيح استعدادي للإجابة على أي أسئلة متصلة بهذه الدراسة ، وقمت فعلاً بالإجابة الوافيه على جميع أسئلته المتعلقة بالدراسة.

توقيع الباحث الرئيسي: ______________________ الاسم (طباعة): د.ليلى أبو طاحون.

التاريخ: ______________________________

تمت مراجعة هذا النموذج والموافقة عليه من قبل مجلس تقييم الأبحاث في مركز الحسين للسرطان

Appendix V

Consent Form for all Participants from JUH or Jordan Hospital

مستشفى الجامعة الأردنية، مستشفى الأردن

نموذج إقرار بالموافقة على البحث المعنون:

العلاقة بين التباين الجيني *M2 في CYP1A1* و سرطان الثدي عند النساء الأردنيات: دراسة مقطعية.

الجزء الأول – معلومات للمشارك في البحث:

ت. <u>الغرض من البحث</u>:

سرطان الثدي هو أكثر أنواع السرطانات انتشارا عند النساء في الأردن. قد تساعدنا هذه الدراسة في فهم أحد أسباب هذا المرض و المتعلقة بحدوث تغييرات في أحد الجينات و هو ما قد يساعدنا في إيجاد علاجات جديدة أو تشخيص المرض في مرحلة مبكرة. يهدف هذا البحث لدراسة العلاقة بين التباين الجيني M2 في CYP1A1 و احتمالية حدوث سرطان الثدي عند النساء الأردنيات

<u>ب. وصف البحث:</u>

تتمثل هذه الدراسة في جمع عينة دم (3مل) من مجموعتين من النساء الأردنيات:

المجموعة الأولى : تتمثل في 100 مريضة مصابة بسرطان الثدي (حاليا أو سابقا)

المجموعة الثانية: تتمثل في 100 امرأة قامت باجراء صورة الماموغرافي و كانت نتيجة هذه الأخيرة سلبية.

عند الانتهاء من تجميع كل هذه العينات سيتم استخراج الحمض النووي منها، و باستخدام تقنية مخبرية اسمها PCR-RFLP سيتم دراسة العلاقة بين الطفرات التي قد تحدث في المورثات الجينية ل CYP1A1 و احتمالية الاصابة بسرطان الثدي.اذا قمت بالموافقة على المشاركة في هذه الدراسة، سيتم سحب عينة دم من الوريد (في المختبر المخصص لذلك)، و ستقوم كذلك الطالبة ايمان عمراني باجراء مقابلة معك للحصول على معلومات مفصلة كالمعلومات الديموغرافية ، والوزن ، والطول ، و بداية دورة الحيض، انتظامها، استمراريتها والحمل ، واستخدام الهرمونات ، التاريخ المرضي، والعادات الغذائية والنشاط البدني، و التدخين، والتاريخ العائلي لسرطان الثدي ، بالإضافة إلى التعرض للإشعاع ، والتعرض للعوامل البيئية،و قياس محيط الخصر و الورك ، و كل ذلك باستخدام نموذج خاص بهذه الدراسة.

ج. <u>مدة مشاركة المريض في هذه الدراسة</u> : ستتم مشاركتك في هذه الدراسة مرة واحدة فقط، خلال الزيارة الروتينية للعيادة أو خلال الزيارة للقيام بفحص الماموجرام

د. <u>عدد المشاركين بالدراسة</u> –: 100 مريضة مصابة بسرطان الثدي و 100 امرأة غير مصابة به.

و. <u>المخاطر والانزعاجات المحتملة</u>: هناك خطر صغير مع عملية سحب الدم ، حيث أن العملية مشابهة لأي

عملية سحب من أجل الفحوص المخبرية: بعض من الألم ، ونادرا كدمات أو التهاب في الموقع الذي تم السحب منه ، وفي بعض الأحيان ، الدوار أو الإغماء. إذا شعرت بالإغماء ، فينبغي عليك أن تعلمي الممرضة.

ز.الفوائد المرجوة :

لن تكون هناك فائدة مباشرة لك عند المشاركة في هذه الدراسة. و لكن من خلال مشاركتك ، قد تساهمين في تحسين العلاج والاكتشاف المبكر لسرطان الثدي عند النساء الأردنيات.

ط. تكاليف المشاركة:

لن تكون هناك أي تكاليف من جراء المشاركة في هذه الدراسة.

بب.المكافآت المالية:

لن تكون هناك أي مكافأات مالية لدى مشاركتك في هذه الدراسة.

ك. المشاركة الطوعية:

المشاركة في هذه الدراسة طوعيه وإذا قررت عدم المشاركة فانك لن تتعرض لأي مضايقات أو لفقدان حقك المشروع في المعالجة.

م. : السرية

كمشارك في هذه الدراسة ستكون هويتك ومحتويات ملفك الطبّي سرية في جميع المنشورات المتعلقة بنتائج الدراسة كما سيبقي رقم سجلك الطبي سري في الحدود التي يسمح بها القانون.

ويمكن الإطلاع على ملفك الطبّي من قبل مجلس تقييم الأبحاث أو الوكيل الداعم للدراسة في حدود النظم والقوانين المطبقة بهذا الخصوص سيتم تزويدك بنسخة موقعة من هذا الإقرار.

ن.: إنهاء المشاركة:

أن قرارك بالانسحاب من الدراسة لن يؤثر على تلقيك للخدمة العلاجية المعتمدة والمتوفرة في مركز الحسين للسرطان.

- **الأشخاص الذين يمكن الاتصال بهم:**

- **في حالة وجود أي استفسار عن الدراسة أو أسئلة محدّدة تتعلّق بهذه الدراسة وفي حالة حدوث أي مضاعفات تتعلق بالدراسة نرجو الاتصال ب أ.د. عبد الله العبادي: 5355000 ، 23453**

Consent form

الجزء الثاني : تفويض وموافقة على الشاركة في الدراسة المعنونة

العلاقة بين التباين الجيني M2 في CYP1A1 واحتمالية حدوث سرطان الثدي عند النساء الأردنييات.

أنا الموقع أدناه ...

وبعد أن شرح لي طبيبي ، ووفقا للنموذج المرفق فانني أوافق وبمحض إرادتي على المشاركة والدخول في الدراسة المتعلقة بالتغير النقطي الذي يحدث في الجينCYP1A1 في مرضى سرطان الثدي كما أوافق على دراسة عينات الدم المأخوذة مني. وأفوض طبيبي لإعطاء مايلزم من المعلومات اللازمة عني وعن مرضي لفريق الدراسة للإستخدامات العلمية اللازمة شريطة أن لا يعطى اسمي في حالة النشر وأستطيع الإنسحاب من الدراسة عندما أشاء وأعلم أن عدم مشاركتي في الدراسة سوف لن يؤثر عليّا سلبيا أو على علاجي أو يكلفني أية تكلفة مالية وعلى ذلك أوقع .

التوقيـــع ....   التاريخ / /

شاهـــــد ....

Appendix VI

Data Collection Form for Patients

Data collection form for the study entitled: Association of *M2* Polymorphism in *CYP1A1* with Breast Carcinoma in Jordanian Women: a Case-control

(Patients Form)

Clinical information (from patient's file)

- Involved breast :
    - Left breast.
    - Right breast.
    - Bilateral
- Stage: --------------------
- Hormone Receptors:
    - ER+/PR-
    - ER+/PR+
    - ER-/PR+
    - ER-/PR-
- HER2:
    - Positive
    - Negative
- Histopathological type:

Data collection form for the study entitled: Association of *M2* Polymorphism in *CYP1A1* with Breast Carcinoma in Jordanian Women: a Case-Control study

*Date: ---

*Patient's Code: --------

*Patient phone number: ---------------------

*Physician: --

*Age ----------------

*Patient's Occupation: -----------------------------------

*Patient's living place ---------------------------------------

*Marital status: Married / Widowed / Divorced / Single

*Education: Illiterate / Elementary school / Middle School/ High school / College / University / Postgraduate.

* Religion (Optional): -------------------------------------

*Race/Ethnicity: ----------------------------

Weight: --------- height: --------, *waist circumference: --------, *hip circumference: -------

*BMI = ---------------

1. How old were you when you have been diagnosed with breast cancer? ____________

2. -Did any of your parents, siblings, or children have breast cancer?
    - Yes
    - No
    - I don't know

3. Who is he (she)?
    - _____________
    - _____________
    - _____________

4. Have you ever been diagnosed with other types of cancer? If yes, which one(s)?
    - Yes, ------------
    - No

5. Income (Optional): Which range of figures comes closest to your total household income for the last calendar year?
    - <250JD
    - >250JD

6. At what age did your menstrual periods begin?
    - <14 years old.
    - 14 or above
    - I don't know

7. Is/Was your period regular?
    - Yes
    - No
8. Do you still have menstrual period?
    - Yes → skip to question 12
    - No

9. When was your last menstrual period? ---------

10. Have you ever been taking hormones for menopause symptoms?
    - Yes.
    - No → skip to question 12.

11. For how long did you receive hormonal therapy? -------------------------

12. Have you ever been pregnant?
    - o Yes
    - o No → skip to question 19.

13. How old were you when your first child was born? -----------------

14. How many times have you been pregnant? -----------

15. Have you ever aborted?
    - o Yes
    - o No, skip to question 17.

16. How many times? ---------

17. Did you breast feed any of your children?
    - o Yes
    - o No → skip to question 19

18. Thinking of all your children, for how many months or years in total did you breast fed them?
    - o First Baby _____
    - o Second _____
    - o Third _____
    - o Fourth ______
    - o Fifth ______
    - o Sixth ______
    - *Seventh______
    - * eighth ______
    - * Ninth _____
    - * Tenth _______
    - * Eleventh _______
    - * Twelfth _______

19. Have you ever used oral contraceptive?
    - o Yes
    - o No →skip to question 22

20. How old were you when you began using oral contraceptive? ---------

21. For how many months or years in total have you used oral contraceptive?
    - o ____ months.
    - o ____ years.
    - o I don't know.

22. Have you participated in physical activities before diagnosis by 5 years?
    - Yes
    - No → skip to question 24

23. How often did you participate in physical activities?
    - < 2 hours per week
    - 2 – 4 hours
    - 5 hours or more
    - I don't know

24. Have you ever smoked?
    - Yes
    - No → skip to question 28.

25. How old were you when you first started smoking cigarettes?
    - Before 20 years
    - After 20 years

26. For how many months/years did you smoke?
    - ____ months
    - ____ years

27. How many cigarettes did you smoke? …… per ……
    - 1 – 10 / day
    - 11 -20 / day
    - > 20 / day

28. Have you been exposed to a direct hit to one of your breasts?
    - Yes
    - No

29. Do you drink alcohol?
    - Yes , regularly
    - Yes, occasionally.
    - No

30. Is your dietary fat intake is high?
    - Yes
    - No

Appendix VII

Data Collection Form from Controls

Data collection form for the study entitled: Association of *M2* Polymorphism in *CYP1A1* with Breast Carcinoma in Jordanian Women: a Case-Control Study

(Controls form)

*Date: -----------------------------------

*Participant's name: ---------------

*Participant's Code: ------------

*Participant phone number: ---------------------

*Physician: ---------------------------------------

*Age ----------------

*Participant's Occupation: -----------------------------------

*Participant's living place ---------------------------------------

*Marital status: Married / Widowed / Divorced / Single

*Education: Illiterate / Elementary school / Middle School/ High school / College / university/Postgraduate

* Religion (Optional): --------------------------------------

*Race/Ethnicity: ----------------------------

*Weight: ------- * height: --------, *waist circumference: ---------, *hip circumference: -------

*BMI = --------------

1. Did any of your parents, siblings, or children have breast cancer?
   - Yes
   - No
   - I don't know

2. Who is he (she)?
   - ______________
   - ______________
   - ______________

3. Have you ever been diagnosed with other types of cancer? If yes, which one(s)?
   - Yes, ------------
   - No

4. Income (Optional): Which range of figures comes closest to your total household income for the last calendar year?
   - <250JD
   - >250JD

5. At what age did your menstrual periods begin?
   - <14 years old.
   - 14 or above
   - I don't know

6. Is/Was your period regular?
   - Yes
   - No

7. Do you still have menstrual period?
   - Yes → skip to question 11
   - No

8. When was your last menstrual period? ---------

9. Have you ever been taking hormones for menopause symptoms?
   - Yes.
   - No → skip to question 11.

10. For how long did you receive hormonal therapy? -------------------------

11. Have you ever been pregnant?
    - o Yes
    - o No → skip to question 18.

12. How old were you when your first child was born? -----------------

13. How many times have you been pregnant? -----------

14. Have you ever aborted?
    - o Yes
    - o No, skip to question 18.

15. How many times? ---------

16. Did you breast feed any of your children?
    - o Yes
    - o No → skip to question 18

17. Thinking of all your children, for how many months or years in total did you breast fed them?
    - o First Baby _____
    - o Second _____
    - o Third _____
    - o Fourth ______
    - o Fifth ______
    - o Sixth ______
    - *Seventh______
    - * eighth ______
    - * Ninth _____
    - * Tenth _______
    - * Eleventh _______
    - * Twelfth _______

18. Have you ever used oral contraceptive?
    - o Yes
    - o No →skip to question 21

19. How old were you when you began using oral contraceptive? ---------

20. For how many months or years in total have you used oral contraceptive?
    - o ____ months.
    - o ____ years.
    - o I don't know.

21. Have you participated in physical activities before diagnosis by 5 years?
    - Yes
    - No → skip to question 24
22. How often did you participate in physical activities?
    - < 2 hours per week
    - 2 – 4 hours
    - 5 hours or more
    - I don't know

23. Have you ever smoked?
    - Yes
    - No → skip to question 27.

24. How old were you when you first started smoking cigarettes?
    - Before 20 years
    - After 20 years

25. For how many months/years did you smoke?
    - ____ months
    - ____ years

26. How many cigarettes did you smoke? …… per ……
    - 1 – 10 / day
    - 11 -20 / day
    - > 20 / day

27. Have you been exposed to a direct hit to one of your breasts?
    - Yes
    - No

28. Do you drink alcohol?
    - Yes , regularly
    - Yes, occasionally.
    - No

29. Is your dietary fat intake is high?
    - Yes
    - No

Appendix VIII

Hardy-Weinberg Equilibrium Calculations

Hardy Weinberg Equilibrium Calculations

Number of Patients with AA (2 alleles of A) = 102

Number of Patients with AG (2 alleles of G) = 10

Number of Patients with GG (2 alleles of G) = 0

So

Number of A alleles = 102 * 2 + 10 * 1 = 214

Number of G alleles = 0 * 2 + 10 * 1 = 10

A allele frequency = 214/224 = **0.955**

G allele frequency = 10 /224 = **0.044**

According to H-W, if the population is in equilibrium, then we will have: $(A+ G)^2 = 1$ so

$A^2 + 2AG + G^2 = 1$ ……………………………………………equation 1

Where A^2 = the predicated frequency of AA in the population, the frequency of AG= 2AG and the frequency of GG = G^2

- Number of patients expected with the AA genotype equals to $(0.955)^2 * 112$ (total number of patients) = 102.
- Number of patients expected with the AG genotype equals to 2 * 0.955* 0.044 * 112= 10
- Number of patients expected with the GG genotype equals to $(0.044)^2 * 112= 0$

With these expected numbers and the observed number, we can Calculate the χ^2 value and the corresponding probability value. The value should be less than 3.14

In the same way, the calculations are done for the control group.

العلاقة بين التباين الجيني *M2* في *CYP1A1* و سرطان الثدي عند النساء الأردنييات: دراسة الحالات الإفرادية المقترنة بحالات ضابطة.

اعداد
ايمان عمراني

المشرف
الدكتورة نائلة بولاتوفا

المشرف المشارك
الأستاذ عبد الله العبادي

ملخص

الجينات المنخفضة النفوذية الزائدة من قابلية الاصابة بالسرطان ، و المتأثرة بالعوامل البيئية و نمط الحياة قد تكون من أكثر المسببات لحالات سرطان الثدي الغير عائلية (المنتشرة). *CYP1A1* هو أحد الجينات المنخفضة النفوذية و المرشحة لزيادة احتمالية الاصابة بهذا المرض و ذلك لدوره المهم في المرحلة الأولى للعمليات الأيضية للمواد المسرطنة كالهيدروكاربونات العطرية المتعددة الحلقات ، بالاضافة لعملية الأيض المؤكسد للاستروجين. هناك العديد من الدراسات التي وجدت علاقة معتبرة بين التباين الجيني *M2 (A4889G)*في جين *CYP1A1* و احتمالية الاصابة بسرطان الثدي بينما دراسات أخرى لم تجد أي علاقة. الهدف الأساسي من هذه الدراسة هو البحث في العلاقة بين التباين الجيني *M2* في *CYP1A1* و خطر الإصابة بسرطان الثدي في النساء الأردنيات و في مجموعات فرعية مقسمة على حسب خصائصها النسيجية والباثولوجية، حالة مستقبلات الهرمونات، التدخين، و الحالة الحيضية، الهدف الثاني هو تقييم التفاعل المحتمل بين التباين الجيني *M2* في *CYP1A1* و عوامل الخطورة الأخرى (كالتدخين، العوامل البيئية و العوامل الهرمونية ... الخ) إن معرفة مثل هذا الارتباط (إن وجد عند النساء الأردنيات) سينتج عنه توصيات من أجل الفحص المبكر و/ أو التقصي بتواتر أكثرعن سرطان الثدي عند الأشخاص الحاملة لمثل هذه الجينات و قد يمثل أيضا هدفا جديدا لتطوير علاجات مستقبلية لهذا المرض. تم سحب عينات دم من 112 مريضة مصابة بسرطان الثدي و 115 مرأة خالية من السرطان (مجموعة ضابطة) و اللاتي قمن باجراء فحص الماموغرافي مرفقا أو لا بتصوير الثدي بالموجات الفوق صوتية، و كانت نتيجة الفحص سلبية. كما أنه قد تمت محاورة المشلركات في الدراسة للحصول على معلومات مفصلة عن العوامل المؤدية لسرطان الثدي كالعوامل الديموغرافية، الهرمونية، البيئية، و عوامل أخرى متعلقة بنمط الحياة. تم تحديد الطبيعة الجينية للجين المذكور سابق عند المشاركات في الدراسة بالستخدام تقنية ال PCR-RFLP. تشير نتائج الدراسة الى أن الطبيعة الجينية تتوزع حسب النسب التالية: من 112 مريضة مصابة بسرطان الثدي، كان هناك 102 (%91.07) *AA* ، 10 (%8.93) *AG* ، 0 (%0) *GG* . و من 115 امرأة من المجموعة الضابطة،

102 (%88.70) *AA* ، 12 (%10.43) *AG*، و 1 (%0.87) *GG*. لم يكن هناك أي ترابط ذو دلالة احصائية بين الطبيعة الجينية للتباين الجيني *M2* في *CYP1A1* و احتمالية الأصابة بسرطان الثدي. (p= 0.55). كذلك أوضح التحليل في المجموعات الفرعية المذكورة سلبقا أنه لا توجد علاقة بين هذا التباين الجيني و سرطان الثدي. الجدير بالملاحظة، أن النشاط الرياضي يقوم بالتقليل من خطر الاصابة بسرطان الثدي بنسبة 0 % خاصة عندالنساء من دون سن اليأس. و لوحظ أيضا أن السمنة بعد سن اليأس و التعرض لأي صدمة مباشرة على الثدي، مرتبطان بازدياد خطر الاصابة بهذا المرض. **الخاتمة**: بناءا على نتائج هذه الدراسة، فاننا نعتقد أن التباين الجيني *M2* في *CYP1A1* ليس عاملا مهما بالنسبة لاحتمالية حدوث سرطان الثدي عند النساء الأردنيات. و لكن بسبب ندرة هذا التباين الجيني عند النساء الأردنيات، فان الدراسات التي تشمل عدد أكبر من النساء ضرورية لتأكيد هذه النتائج أو رفضها.